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Begin the article with a short summary.

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Submit each table on a separate page, typed double spaced. Please use standard abbreviations; otherwise spell out.

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Symposium on "Sarcomas of Soft Tissue and Bone in Childhood"
Second International Conference on the Adjuvant Therapy of Cancer

The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1980.

COMMENTARY

Cell Kinetics and Chemotherapy: A Critical Review [1]

Ian Tannock [2,3,*]

SUMMARY

The paper reviews methods of studying cell kinetics in man, cell population kinetics of human tumors and bone marrow, drug interactions and the cell cycle, and possible applications to chemotherapy. The conclusions drawn are: (1) Cell cycle time and S-phase duration for proliferating granulocyte precursors in human bone marrow are poorly defined but are probably shorter than median values for most human tumors, including leukemia. (2) Most drugs have greater toxicity for cycling cells and some variation in toxicity at different phases of the cell cycle. There is a special need for chemotherapy directed at slowly proliferating and hypoxic tumor cells. (3) Pretreatment indices of tumor cell kinetics are of little value in choosing drugs or in predicting response. (4) Experiments in animals have demonstrated that therapeutic index may depend on schedule. Knowledge of cell kinetics in animals rarely allows prediction of the optimal schedule and is unlikely to do so in man. Optimal schedules in mice are not directly relevant to man. (5) Measurement of tumor labeling index or DNA histogram by flow microfluorimetry to detect cell synchrony is of little benefit in scheduling if concurrent changes in bone marrow are ignored; these methods are invalid at short intervals after treatment because surviving clonogenic cells are indistinguishable from a larger number of drug-damaged cells prior to their lysis. (6) The major factor determining the outcome of chemotherapy is the availability of drugs with activity for the tumor and acceptable host toxicity. Claims that complex schedules using several drugs are effective because of synchrony or kinetic differences of tumor and normal tissue are at present unsubstantiated.

[Cancer Treat Rep 62:1117–1133, 1978]

Improvements in cancer chemotherapy require greater specificity for the killing of tumor cells compared with those of normal tissues. Target cell specificity and differences in drug uptake and metabolism are major mechanisms underlying therapeutic effectiveness and usually determine the choice of drugs for a particular tumor.

Optimal scheduling of drugs might also improve therapeutic index (TI). Many drugs act selectively at one phase of the cell cycle and cause partial synchrony of surviving cells; other agents are less phase specific but have greater toxicity for rapidly proliferating cells. Scheduling of drugs to take advantage of differences in cell proliferation of tumor and critical normal tissues might therefore improve tumor cell-kill specificity. Enthusiasm for this approach has varied from euphoria to despair.

I shall review cell population kinetics of human tumors and critical normal tissues and the relationship between drug activity and the cell cycle. I shall then try to assess the clinical potential of cell kinetics in cancer chemotherapy.

[1] Received Sept 20, 1977; revised Mar 8, 1978; accepted Mar 15, 1978.

[2] Department of Medicine, Ontario Cancer Institute Incorporating The Princess Margaret Hospital, Toronto, Canada.

[3] I wish to thank Drs. D. Bergsagel and R. P. Hill for their advice, and Dr. G. G. Steel for computing the PLM curves of figures 2 and 3.

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CELL PROLIFERATION: TERMINOLOGY AND METHODS

A model of a cell population is shown in figure 1. The proliferating cells have cycle time T_c, and the duration of constituent phases of the cell cycle is T_i, where i is the appropriate subscript. Phase duration (particularly T_{G1} and T_c) may vary widely among the cells of the population.

Many normal tissues and tumors contain noncycling but living cells. Growth fraction (GF) is the ratio of proliferating to total cells (1). The nonproliferating population may include cells that are incapable of producing new cells, but also cells that could divide if stimulated (fig 1). In normal tissues clonogenic but nonproliferating cells are a reserve or "G_0 population." The stimulus for cell division may be mediated through systemic or local hormones (eg, erythropoietin) released in response to a requirement for new cells. Tumor cells may also retain proliferative potential when noncycling, and such cells are an important target of chemotherapy. Renewed proliferation can be stimulated by a change in the cell environment and may cause tumor regrowth after drug-induced death of more sensitive cycling cells (2–5).

Thymidine Labeling and Autoradiography

After injection of tritiated thymidine (^{3}H-TdR) into animals or man, the isotope is incorporated into cell nuclei that are synthesizing DNA, while the remainder is degraded and excreted rapidly. Thus, the number of cells incorporating ^{3}H-TdR is similar to the number of cells synthesizing DNA at the time of injection. Labeled cells can be recognized in autoradiographs of sections or smears of tissue. The resolution is good because β particles from tritium have short range and grains are localized above the cell nucleus. The following techniques are used to estimate cell cycle parameters.

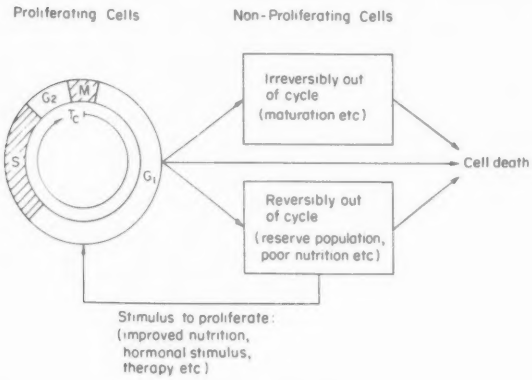

FIGURE 1.—Model of a cell population.

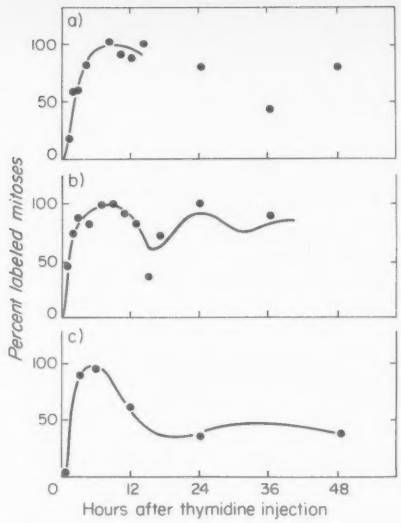

FIGURE 2.—PLM data and computer-determined curves for granulocyte precursors in human bone marrow. *a.* Data for a patient with normal hematopoiesis from Stryckmans et al (41). The experiment does not define a second wave of labeled mitoses. *b.* Data for a patient with mild polycythemia vera [from Stryckmans et al (41)]. Computed median values are T_s = 13 hrs and T_c = 16 hrs. *c.* Data for a patient with normal hematopoiesis [from Todo (42)]. Computed median values are T_s = 12 hrs and T_c = 25 hrs.

Labeling index (LI).—LI is the ratio of labeled cells (N_s) to total cells (N) in autoradiographs of tissue removed shortly (usually 1 hour) after injection of ^{3}H-TdR. LI has been estimated for human tissue by preparing autoradiographs after short incubation of fresh biopsy specimens with ^{3}H-TdR in vitro. There is good correlation between mean values of LI estimated for tissues of experimental animals by in vivo and in vitro labeling (6,7). Comparison of values of LI for different tissues can give a rough comparison of rates of cell proliferation (8); comparison of values of LI in the same tissue before and after chemotherapy may indicate changes in the proliferative population (4,5).

Doubling labeling (DL).—DL provides a method for estimating T_s (9,10). Two isotopic labels are used, and injections of ^{3}H-TdR and ^{14}C-TdR are commonly given with a known short interval of 1–2 hours between injections. Tissue is excised shortly after the second injection. Cells labeled with ^{14}C($\pm^3$H) are recognized in autoradiographs by a halo of grains around the cell generated by the higher energy β emission from ^{14}C; cells labeled with ^{3}H alone are

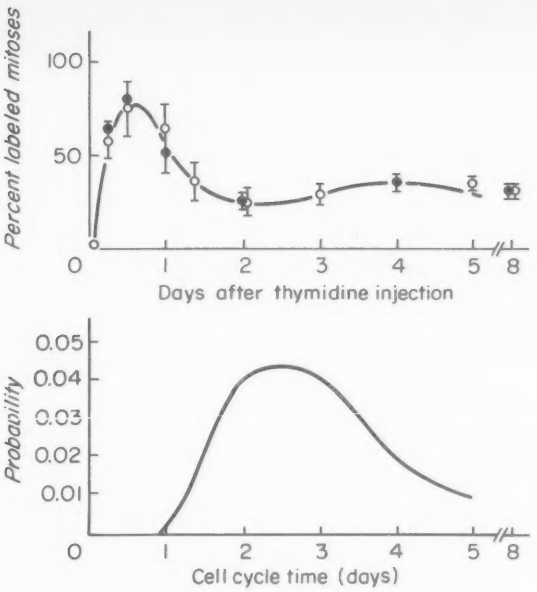

FIGURE 3.—PLM data for malignant melanoma [from Shirakawa et al (74)]. ○ and ● = results from 2 patients. A computer-determined PLM curve is fitted to the points, and the computed distribution of cell cycle times is shown in the lower figure.

recognized by the presence of grains over the nucleus only. T_s may then be calculated from:

$$\frac{T_s}{t_i} = \frac{\text{No. of cells labeled with } ^{14}C(\pm\ ^{3}H)}{\text{No. of cells labeled with } ^{3}H \text{ alone}} \text{ (9,10).}$$

Resolution of cells labeled with ^{14}C and with ^{3}H may be easy in smear preparations where cells are spread thinly or in sections of slowly proliferating tissue where few cells are labeled. It is almost impossible in sections of rapidly proliferating tissue. Autoradiographic technique is critical or confusion of ^{14}C- and ^{3}H-labeled cells will occur, leading to an underestimation of T_s (10). The method has been adapted to human tissue by incubating fresh biopsy specimens with either the second isotope or both isotopes in vitro (11–16). However, many authors have not justified their in vitro method by comparing in vivo and in vitro results in experimental animals.

Percent labeled mitoses (PLM).—PLM is a powerful method for estimating mean duration and variability of the cell cycle and its constituent phases. Tissue is excised at intervals after a single injection of ^{3}H-TdR. Autoradiographs are prepared and mitotic figures are scored as labeled or unlabeled; a curve is generated by plotting PLM against time after thymidine administration (figs 2 and 3). Only cells in S phase at the time of injection are labeled; in the absence of variability in phase duration, waves of labeled mitoses of width T_s and separation T_c are generated each time the labeled cohort of cells passes through mitosis. Variability in phase duration leads to damping of the curve, and damping tends to be considerable when human tissue is studied. The PLM curve may be analyzed by computer to give a mean and distribution for each phase duration and for cell cycle time (fig 3). Several computer programs are available (17,18), and the choice is arbitrary since the PLM method is limited usually by the quality of experimental data rather than by the model used to fit the curve. The PLM technique is the only method available for measuring duration of all phases of the cell cycle. Its main disadvantages are that multiple biopsies are required and scoring of mitotic figures is laborious in slowly proliferating tissue.

Estimation of Growth Fraction

A PLM curve and an estimate of LI together allow calculation of GF. GF is equal to the ratio of the measured LI to the value of LI that would be obtained by sampling only proliferating cells. The expected LI for proliferating cells may be obtained from the level of damping of the PLM curve at long intervals after ^{3}H-TdR injection (1) or by direct computation from the distribution of values of T_s and T_c (17). These methods for estimating GF are potentially useful in defining a population of cells that might be sensitive to cycle-specific chemotherapy. The estimate of GF is, however, a property of the analytic model rather than of the cell population since the techniques are not sufficiently sensitive to allow separation of truly nonproliferating from proliferating cells with cycle times much longer than the mean.

An alternate method of estimating GF assays cells for the presence of DNA-dependent DNA-polymerase, an enzyme presumed to be specific for proliferating cells (19,20). The uptake of ^{3}H-thymidine-5′-triphosphate is measured autoradiographically in dried nuclei obtained from single cell suspensions of tumors. Good correlation has been obtained with estimates of GF by other methods (20), but reproducibility between laboratories has yet to be assessed.

Automated Methods for Studying Cell Kinetics

Autoradiographic methods are time-consuming. They have been applied to selected patients and

have added greatly to the knowledge of human biology, but they are not practical for use as part of a pretherapy assessment of the general cancer patient. Rapid automated techniques are under study in several laboratories.

Uptake of ^{3}H-TdR may be assessed rapidly by scintillation counting (21); unfortunately, thymidine availability and uptake by individual cells often vary markedly so that comparison of scintillator counts may not reflect a comparison of proliferative rate.

Flow microfluorimetry (FMF) is an automated method for estimating DNA content among cells in single cell suspension and can produce DNA distributions for up to 10^5 cells/minute (22,23). The DNA histogram may be analyzed to provide similar information to LI, but because diploid G_1 and nonproliferating cells cannot be separated, it does not allow estimates of phase duration or GF. In patients with lesions allowing repeated biopsy, the method might give a rapid indication of drug-induced synchrony (23), although it cannot distinguish drug-damaged cells prior to their lysis from true surviving cells. The FMF method cannot detect cells of different morphology, may be complicated by aneuploidy, and like all methods involving cell suspensions, may be subject to error from inclusion of degenerating or pyknotic cells.

CELL PROLIFERATION IN MAN

Ethics of Human Experimentation

Knowledge of the cell cycle in human tissue derives largely from experiments in which one or more biopsies have followed injection of ^{3}H-TdR. The isotope is incorporated into DNA of replicating cells, and since the penetration of emitted β particles is 1–2 μ, the greatest risk is of genetic mutation. Malignant change and birth defects in subsequent offspring are possible serious side effects. There have been no reported toxic effects from the use of ^{3}H-TdR in man, and in patients with cancer the risks are probably small compared with those from the disease or its treatment (24). The method cannot be justified in healthy people. Suitable subjects undergo the pain of biopsy procedures without immediate benefit to themselves, and informed consent is mandatory.

Bone Marrow

Hematopoiesis derives from uncommitted and unrecognizable stem cells. In mice, the spleen-colony assay has demonstrated a common precursor (CFU-S) to granulocytic and erythroid cells (25). CFU-Ss are resistant to high doses of ^{3}H-TdR and to cycle-specific drugs, and apparently have a low rate of proliferation (26,27). There is no corresponding assay in man, but studies of G6PD isoenzymes in myeloproliferative syndromes infer a common precursor of granulocytes and erythrocytes (28).

Murine and human bone marrow can now be cultured in vitro. Stem cells which generate colonies in culture (CFU-C) differ from CFU-S in that they produce only cells of the granulocyte series and their rate of proliferation is more rapid (29,30). About 20%–25% of CFU-Cs in normal human marrow are inactivated by a dose of ^{3}H-TdR lethal to cells in S phase (30). Human CFU-Cs and murine CFU-Ss may increase their rate of proliferation after stimuli such as endotoxin, androgens, and anticancer drugs (30–33).

Dose-limiting toxicity of anticancer drugs probably results most often from effects on committed granulocyte and platelet precursors. Estimates of LI for granulocyte precursors have ranged from 30%–75% (mean, 43%) for myeloblasts to about 20% for myelocytes, implying a rapid rate of proliferation (34–40). Cell cycle time and phase duration for granulocyte precursors are poorly defined. An estimate of T_s by DL gave a low value of 5–6 hours (11) but this is probably an underestimate due to artefacts in the method. PLM curves have been derived for granulocyte precursors in only two patients with normal bone marrow and in only one patient with mild polycythemia vera (41,42). These data are shown in figure 2, and best-fit PLM curves have been computed by the method of Steel and Hanes (17). The data of figure 2a fail to define values of T_s and T_c; computed estimates of median values are T_s = 13 hours and T_c = 16 hours (fig 2b), and T_s = 12 hours and T_c = 25 hours (fig 2c), but the shape of the PLM curve is poorly defined beyond the first wave in figure 2c. Clearly, there is need for more information about the cell cycle of granulocyte precursors in man.

Erythroid precursors also have a rapid rate of proliferation. Estimates of LI range from 30% to 75%, with higher values for earlier cells in the maturation sequence (12,38,39). Four PLM curves have been analyzed and the results are similar to those for granulocyte precursors (41,42). The median range for T_s is about 13 hours, while the median values for T_c are in the range of 16–26 hours. An estimate of T_s by DL gave a value of 18 ± 2 hours with a maximum estimate of T_c = 23 ± 2 hours; corresponding values in patients with anemia secondary to thalassemia were T_s = 10 hours and T_c = 13 hours (12). Erythroid precursors are probably as sensitive to chemotherapy as their granulocytic

counterparts, but anemia is rarely dose-limiting because of the long lifespan of the rbc and the availability of blood transfusions.

Little is known of the kinetics of other cells in human bone marrow: monocytes, lymphocytes, plasma cells, and megakaryocytes. Drugs may be toxic to all of these cells but only thrombocytopenia is commonly dose-limiting. Megakaryocytes are probably derived from pluripotential stem cells but then undergo periods of DNA synthesis followed by separation of the chromosomes without cell division, resulting in polyploid cells. Rat megakaryocytes have a mean LI of 73% for 4N cells decreasing to 14% for 32N cells, while PLM curves give values of T_s = 7.6 hours and T_c = 9.3 hours (interval between successive chromosomal separation) (43,44). No data are available in man, but a high rate of DNA synthesis and chromosome separation is likely.

Nonhematologic Tissues

Damage to rapidly proliferating tissue other than bone marrow is observed after cancer chemotherapy. Alopecia and sterility may occur but cannot be considered dose-limiting in the treatment of potentially lethal disease. Gastrointestinal toxicity may be dose-limiting. Nausea and vomiting often occur acutely but are due to effects of drugs on the central nervous system; more important is mucosal ulceration which can follow administration of methotrexate, 5-fluorouracil (5–FU), or other drugs. PLM curves have been obtained for lower parts of the human gastrointestinal tract by a biopsy of the rectum, a colostomy, or a peroral tube. Cells in intestinal crypts have values of LI = 12%–18%, T_s = 11–18 hours, and mean T_c = 24–48 hours (45,46). This rapid turnover of gastrointestinal epithelium is consistent with the observed sensitivity to some cycle-specific drugs.

Dose-limiting toxicity of some drugs (eg, neurotoxicity of vincristine, cardiotoxicity of adriamycin, and pulmonary and skin toxicity of bleomycin) is probably not related to the cell cycle. Study of cell proliferation of other normal tissues is biologically important but is not relevant to the potential improvement of cancer chemotherapy through better drug scheduling.

Leukemia

Values of LI for untreated leukemia, estimated by in vivo and in vitro methods, are listed in table 1 and are lower than values of LI for granulocyte and erythroid precursors in normal bone marrow. Thus, accumulation of cells in leukemia is not due to an increased rate of proliferation. Instead, there is a failure of maturation and the population of leukemic cells increases because the rate of cell proliferation (although slower than that of normal myeloblasts) exceeds the rate of cell death or removal from the population.

Mean values of T_s and T_c for untreated leukemia

TABLE 1.—Mean LI values for human leukemia*

Type of leukemia	Cell type	Range of mean values of LI (%)	Ref No.
Acute myelogenous	Myeloblasts, marrow	5–11	35–37
	Myeloblasts, blood	3–9	47–51
Acute lymphoblastic	Lymphoblasts, marrow	4–12	37,46
	Lymphoblasts, blood	2–10	50–53
Chronic myelogenous	Myeloblasts	9–43	36,40
	Myelocytes	8–27	54,55
	Myeloblasts, blast cell crisis	6–18	47,54,55
Chronic lymphocytic	Large lymphocytes	0–1	56
	Small lymphocytes	0.0	
Normal bone marrow	Myeloblasts	32–75	30,34–40
	Myelocytes	18–25	
	CFU-C (thymidine suicide method)	20–25	

*Values for normal granulocyte precursors are included for comparison.

are listed in table 2. Most of these data were obtained directly from published PLM curves, and some estimates differ slightly from those of the original authors. Many of the PLM curves had damped second waves implying wide variability of cycle time about the mean. Mean values of T_s and T_c for leukemia (about 20 and 60 hours respectively) are longer than the best available estimates for granulocyte and erythroid precursors in nonleukemic marrow. GF is equal to the ratio of measured LI (commonly about 10%, table 1) and the estimated LI for cycling cells (commonly about 30%, based on data in table 2). A representative value of GF in human leukemia is about 30%.

In acute leukemia large blast cells have a high value of LI (30%–70%) and are probably all in cycle, whereas small blast cells have a value of LI close to 0 (2,3,42,58). Small blast cells take up label slowly during continuous infusion of ^{3}H-TdR (65) and probably are derived from larger rapidly proliferating cells. Small blast cells also re-enter the cycle and replenish the population of larger cells (2,3,58). Thus, small blast cells may be a slowly proliferating reserve population of leukemic cells. The GF measured in labeling experiments consists of the rapidly proliferating larger cells, but many of the small blast cells probably retain their capacity for proliferation and are important targets for chemotherapy.

Solid Tumors

In vitro estimates of LI are available for many solid tumors in man; values from two reviews and from larger individual series are shown in table 3. In one review the median value of LI for 170 various solid tumors was 3%. Despite this low value, the corresponding rate of cell production was much greater than the measured growth rate of most human solid tumors, implying a high rate of cell death (8).

Detailed studies of the cell cycle in solid tumors have been limited to sites that allow multiple biopsies. A representative PLM curve for two patients with malignant melanoma is shown in figure 3 (74). Like most published PLM curves it has a damped second wave implying variability in values of T_c among individual cells (fig 3). Mean values of T_s and T_c obtained for human tumors by the PLM or DL methods are listed in table 4. Where possible, these estimates were obtained directly from experimental data and may differ from those listed by the original authors. A few published data were probably subject to artefact or misinterpretation and have been excluded from table 4. An example is the unusually low estimate for mean T_c (14–16 hours) quoted in a study of carcinoma of the cervix (89). In this study a scatter of points about the descending limb of a first wave of labeled mitoses may have been misinter-

TABLE 2.—Mean T_s and T_c values for human leukemia*

Type of leukemia	No. of patients	Technique	Mean T_s† (hrs)	Mean T_c† (days)	Ref No.
Acute myelogenous	1	PLM	20	—	42
	2	PLM	19–22	2.5–3.0	48
	1	PLM	20	2.5	57
	1	PLM	16	—	58
	3	PLM	14–17	1.5–2.0	59
		DL	14–16	—	
Acute lymphoblastic	1	PLM	19	3.5	53
	2	PLM	24–28	3.0	57
	1	PLM	18	2.0–3.0	60
	5	PLM	24–42	2.0–3.0	61
Chronic myelogenous (all stages)	1	PLM	20	—	42
	3	DL	14	—	62
	5	PLM in vitro	20–26	2.0–2.5	63
Erythroleukemia	1	PLM	17	—	42
Plasma cell leukemia	1	PLM DL	17	2.5	64
Granulocyte precursors in nonleukemic marrow	2	PLM	12,13	0.7–1.1	41,42

* Values for granulocyte precursors in nonleukemic marrow are included for comparison.

† Mean T_s and T_c values were estimated directly from published data and may differ slightly from estimates of original authors. Mean T_c is expressed to the nearest 0.5 day for leukemia, since data do not define a more accurate value.

preted as evidence for a very narrow second wave. The data can be reinterpreted to suggest a mean value for T_s of about 16 hours, with insufficient data to estimate T_c.

Cells in many human tumors have a short value for mean T_c of 2–4 days (table 4) but a low value for LI; it follows that the GF is usually low. For example, patients with malignant melanoma whose PLM curve is shown in figure 3 had a mean value for LI of about 5%, consistent with a value of GF close to 20% (74).

The rate of cell proliferation is often variable in solid tumors, and values of LI tend to be greatest in well-nourished peripheral regions (89, 90). In animal tumors, the values of LI and mitotic index may decrease rapidly with increasing distance from a capillary, implying that the rate of cell proliferation is sensitive to the nutrient supply from the blood (91, 92). Also, the rate of cell proliferation tends to decrease as experimental tumors grow larger (93–95), perhaps because of decreasing vascularity and nutrition (92). These results have implications for the (adjuvant) treatment of micrometastases; cells in very small tumors may be rapidly proliferating and sensitive to chemotherapy.

DRUG ACTIVITY AND THE CELL CYCLE

Clonogenicity and Cell Survival

Useful anticancer drugs cause sufficient injury to tumor cells that they lose their ability to regenerate the tumor. Measurement of cell survival requires exposure of cells to a drug, followed by an assay for the number of cells which are capable of generating viable clones. Cell survival is estimated most easily by measuring the ratio of numbers of colonies generated from drug-treated and untreated cells after plating in tissue culture. In vivo cloning assays for bone marrow stem cells and for tumor cells are also available in experimental animals (25,26). Cloning assays have demonstrated that cell killing usually follows first-order kinetics so that, over a certain range of drug dosage, cell survival is exponentially related to drug dose (fig 4a).

Drug Activity and Proliferative Rate

Bruce et al (26) used the spleen-colony assay to compare the survival of slowly proliferating bone marrow CFU-S and a rapidly proliferating mouse

TABLE 3.—Representative in vitro LI values for solid tumors in man

Site	No. of patients	Mean or median LI (%)	Ref No.
Squamous cell carcinoma (various sites)	68	8.3	66
Adenocarcinoma (various sites)	121	2.1	66
Sarcoma (various sites)	32	3.8	66
Lymphoma	9	32.0	8
	15	29.2	66
Multiple myeloma	13	2.4	67
Brain	15	2.0	8
Lung and larynx	8	15.0	8
	11	5.5	68
	55	7.6	69
Breast	38	1.1	8
	170	1.9	69
	66	3.0	70
Colon	31	4.5	8
	134	3.1	69
Bladder	70	3.6–24.2*	71
Cervix and uterus	6	4.8	8
	7	18.3	72
	13	17.3	73

* LI values increase with histologic grade.

lymphoma after in vivo treatment with various drugs. Two types of survival curves were obtained. For alkylating agents and 5-FU, survival was exponentially related to drug dose, but many drugs had greater sensitivity for rapidly proliferating lymphoma cells (fig 4a). For drugs such as vinblastine or methotrexate, now thought to be phase specific, survival decreased exponentially up to a certain dose, but then remained constant with further increases in dose (fig 4b). The plateau level of survival was lower for lymphoma cells; presumably, more of the rapidly proliferating lymphoma cells passed through the drug-sensitive phase during the period of drug exposure (26). An increase in drug dosage with a short exposure time gave no further cell-kill because all cells in the drug-sensitive phase of the cycle were killed and no others were affected. However, an increase in exposure time with constant drug concentration allowed more cells to enter the drug-sensitive phase and survival was exponentially related to exposure time (96).

Differences in intrinsic drug sensitivity of lymphoma and bone marrow CFU can be eliminated by adapting the spleen-colony method to assess survival of bone marrow CFU-Ss that have been stimulated to proliferate prior to drug exposure. Rapid proliferation is induced by transplantation into supralethally irradiated mice (97,98) or by treatment with endotoxin (99). An assay for survival of rapidly and slowly proliferating lymphoid cells is also available (100).

Many anticancer drugs have been tested against cells of different proliferative rate by one of the above methods. Drugs which are entirely cycle specific and exhibit no further cell-kill above a threshold dose include most of the antimetabolites: vinblastine, vincristine, methotrexate, cytosine arabinoside, and 6-mercaptopurine (26,96–98,100). Procarbazine also has no increase in toxicity above a threshold dose when tested against CFU-S in mouse bone marrow and, like antimetabolites, is much more toxic to stimulated, rapidly proliferating CFU-

TABLE 4.—Mean T_s and T_c values obtained for solid tumors in man

Type of tumor	No. of studies	Technique	Mean T_s* (hrs)	Mean T_c* (days)	Ref No.
Skin					
Squamous cell	2	PLM	12	1.5–2.0	75
Basal cell	3	PLM, DL	19–20	—	75–77
Melanoma	7	PLM, DL	14–21	2.0–3.0	74,78–80
Breast	3		19–24	1.5–2.0	78
	1	PLM	13	—	80
	1		18	3.0–4.0	81
	1 (8 patients)	PLM (composite)	24	~3.0	82
	29	DL	22±4	—	83
Squamous cell carcinoma, head and neck	1	PLM	11	1.0	80
	4		28–28	2.5–3.0	84
Lung and larynx	5	PLM, DL	19–23	—	13,85
	1	PLM	6	—	80
	1	PLM	17	4.0–5.0	81
Colon and rectum	8	DL	15–22	—	16
	10		17	2.5–3.0†	86
Neuroblastoma	1	PLM	27	—	87
Glioma	11	DL	5–13	—	15
Schwannoma	1	PLM	17	1.5–2.0	80
Lymphomas					
Hodgkin's disease	4	DL	8–13	—	14
Lymphocytic	1		14	—	
Histiocytic	2	DL, PLM	8, 14	2.0	14,88

* Mean T_s and T_c values were estimated directly from published data and may differ from estimates of original authors. Mean T_c is expressed to the nearest 0.5 day, since data do not define a more accurate value. Many PLM curves are well-defined only over the first wave and allow estimates of T_s but not T_c.

† Estimated mean value of T_c from estimate of GF by labeled DNA-polymerase method.

S (98). Drugs for which survival is exponentially related to dose are listed in table 5, together with their relative sensitivity for rapidly and slowly proliferating cells (ie, the ratio of slopes of survival curves). Most drugs have greater toxicity for rapidly proliferating cells.

In vitro assays also are available for studying drug activity against cells of different proliferative rate. Cells in culture grow exponentially when conditions are ideal; however, as cell density increases, proliferation slows because of an increase in cell cycle time and a decrease in GF (106,107). Cell death increases to equal the rate of cell production, and the number of cells reaches a maximum plateau level. Survival curves may be compared for rapidly proliferating cells exposed to a drug in exponential phase and for slowly proliferating cells exposed in plateau phase. Most drugs have greater sensitivity for rapidly proliferating cells in exponential phase (108–111). Some exceptions include the nitrosoureas and bleomycin, which in some (but not all) cultures have equal or greater toxicity against slowly proliferating plateau-phase cells (111–115).

The GF in solid tumors is often low. Noncycling but probably clonogenic cells are often situated distant from tumor blood vessels where the concentration of oxygen and other metabolites is low (91,92). Such cells are resistant to radiotherapy because of hypoxia; they may be resistant to drugs because they are slowly proliferating, because drug

TABLE 5.—Anticancer drugs for which cell survival is exponentially related to dose*

Drug	Assay system	Relative activity†	Ref No.
Mechlorethamine	Lymphoma and CFU-S	1.0	26,101
	Lymphoid cells	1.4	100
	CFU-S	1.6, 1.0	98,102
	CFU-C	1.0	102
Melphalan	Lymphoma and CFU-S	2.6	101
	CFU-S	2.5, 1.0	98,102
	CFU-C	1.0	102
Busulfan	CFU-S	1.7	98
Chlorambucil	Lymphoma and CFU-S	4.6	101
	CFU-S	1.8	98
Cyclophosphamide	Lymphoma and CFU-S	10–15	26,101
	Lymphoid cells	5	100
	CFU-S	2.3, 2.0	98,102
	CFU-C	4.1	102
BCNU	Lymphoma and CFU-S	12	101
	CFU-S	1.5–3.3, 1.2	98,102
	CFU-C	1.2	102
CCNU	CFU-S	1.6	98
Methyl-CCNU	CFU-S	1.3	98
DTIC	Lymphoma and CFU-S	8	101
	CFU-S	1.6	98
5-FU	Lymphoma and CFU-S	6	26
	Lymphoid cells	Infinite‡	100
	CFU-S	4	98,103
	CFU-C	4	102
Actinomycin D	Lymphoma and CFU-S	6	26
	Lymphoid cells	1.8	100
Adriamycin	Lymphoma and CFU-S	6.3	104
	Leukemia and CFU-S	7.5	105
Daunorubicin	Lymphoma and CFU-S	3.6	104
Bleomycin	CFU-S	10	99

*Relative activity against rapidly and slowly proliferating cells.
†Ratio of slopes of survival curves.
‡5-FU had no activity against slowly proliferating lymphoid cells.

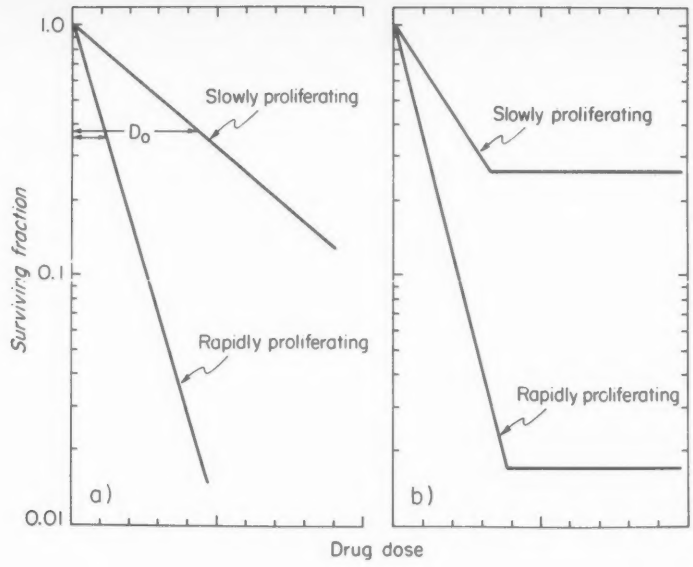

FIGURE 4.—Model cell survival curves for rapidly and slowly proliferating cells (26). a. Continuing exponential decrease in cell survival is observed for many drugs including alkylating agents and 5-FU. Rapidly proliferating cells are often more sensitive than slowly proliferating cells. b. Plateau level of cell survival with no increase in cell-kill with increase in dose above a certain value is observed for many phase-specific drugs. More rapidly proliferating cells enter the sensitive phase and the plateau level of survival is lower than that for slowly proliferating cells.

concentration in their vicinity is low, or because oxygen and other metabolites are required for drug uptake and activity. There is a special need for therapy designed to attack slowly proliferating and hypoxic cancer cells. Possible alternatives to conventional anticancer drugs are nitroimidazoles such as metronidazole or misonidazole. These drugs and others (eg, 5-thio-D-glucose) have specific toxicity for hypoxic cells (116–118); they are likely to have minimal effects when used alone, but might act synergistically with drugs that kill well-oxygenated and proliferating cells.

Phase Specificity

Drug activity against cycling cells may be separated into phase-dependent lethal effects and nonlethal delay of progression through the cell cycle. Information about phase specificity comes largely from experiments with synchronized cells in tissue culture, and data for many drugs have been reviewed by Madoc-Jones and Mauro (119).

Almost all drugs show variation in lethal toxicity around the cell cycle. Cytosine arabinoside, 6-thioguanine, and hydroxyurea are probably S-phase specific (119–123); methotrexate, 5-fluorodeoxyuridine, adriamycin, and daunorubicin have maximum toxicity for S-phase cells but have some activity at other phases of the cycle (119–121,124–126). Many of these drugs also inhibit either the onset or continuation of DNA synthesis in surviving cells. Surprisingly, vinblastine and vincristine often exert maximum lethal toxicity in S phase, although they cause progression delay in mitosis (119). Many alkylating agents have similar phase activity to radiation with two peaks of maximum toxicity, one in mitosis and one near the G_1/S boundary (119,120), although results with the nitrosoureas seem to depend on the cell line studied (119,120,127,128). Bleomycin acts mainly in G_2 and mitosis (119,126,127). Among commonly used drugs, only 5-FU, steroids, and *cis*-dichlorodiammineplatinum(II) have rather uniform activity around the cell cycle (119,120,129).

APPLICATIONS TO THERAPY

The increased sensitivity of proliferating cells to some chemotherapeutic agents is but one of the possible mechanisms to be exploited in achieving increased tumor cell-kill specificity. If the drug does

not fit the target site, if the drug-transport system is blocked, or if the tumor cell cannot activate the drug or inactivates it too rapidly, the proliferative state of the cell does not affect cell-kill specificity. In general, rapidly growing tumors such as acute leukemia, and some lymphomas, pediatric tumors, and testicular tumors, are more sensitive to chemotherapy than slowly growing tumors. However, effective drugs for rapidly growing tumors include agents of low cycle specificity such as alkylating agents and bleomycin, while in contrast the best agent for slowly proliferating squamous cell tumors of the head and neck is probably methotrexate. The rate of proliferation within a tumor is of little help in choosing the drug to treat it.

Prognostic Value of Labeling Index

The relationship between pretreatment LI of the tumor and response to chemotherapy is complex. A correlation of favorable response to treatment with a higher than average LI has been demonstrated in some studies of acute leukemia (130,131), carcinoma of the breast (132), and a variety of solid tumors (133). In the last study the mean LI of responders was twice that of nonresponders in a group of 198 patients. Many drugs were used, but the leukemia patients received cytosine arabinoside either alone or in combination.

There was no correlation of pretreatment LI and response to therapy in a large variety of solid tumors treated with 5-FU (69), and frequency of complete remission (CR) was independent of pretreatment LI in another study of 58 adults with acute leukemia (134). Remissions in acute leukemia tend to be of shorter duration in patients with a high pretreatment value of LI (131,134).

Success of therapy depends on a balance between cell-killing by drugs and proliferation of surviving cells. Both processes are likely to be rapid in tumors with a high rate of cell proliferation; thus, pretreatment measurement of LI or other kinetic parameters is of limited prognostic value.

Application of Experimental Studies to Scheduling

A large amount of data are available on scheduling of drugs in experimental tumors, most of it derived from ascites tumors such as L1210 leukemia. The results of these experiments may be broadly summarized as follows:

1. Phase-specific drugs have minimal effects when given as single doses, but may be highly effective after repeated administration (135–137).

2. Therapeutic benefit (measured for example by increase in lifespan of tumor-bearing animals) is often dependent on the interval between applications of a drug (135,137,138).

3. When two drugs exert major activity in the same phase of the cell cycle, therapeutic benefit usually accrues from giving them at separate times rather than simultaneously (139–141), although exceptions are reported (142).

4. Small tumors have a higher rate of cell proliferation than large tumors and are more frequently cured by chemotherapy (136,137,143–145).

The above results have laid some foundations for human cancer chemotherapy. Phase-specific drugs are usually given in multiple doses or by continuous infusion, while the good response of small animal tumors supports the clinical use of adjuvant chemotherapy for occult metastases.

Animal studies have not been helpful in suggesting optimal schedules in man. Direct extrapolation of optimal schedules in mice would be unlikely; animal tumors in general, and L1210 leukemia in particular, tend to be rapidly growing and homogeneous, while human tumors tend to be slow-growing and heterogeneous with respect to phase duration and other parameters.

A more serious limitation of results of scheduling in animals is that optimal schedules are usually found empirically; the optimal schedule could be predicted in few experiments, despite a fairly complete knowledge of tumor cell kinetics. An example is the treatment of L1210 leukemia with cytosine arabinoside. In two laboratories the best therapeutic response was obtained with a dose interval of about 3 hours (137,138), whereas maximum synchrony of tumor cells in S phase occurred at a longer dose interval of 8–10 hours, closer to the mean value of T_s for tumor cells (138). Greater killing of tumor cells could be obtained by using a schedule with this longer dose interval, but there was greater toxicity (138,146) and a lower TI.

Potential benefit to be gained from scheduling depends on the relative effects on tumor and normal tissue. If cell kinetics has relevance to scheduling, it is the *difference* in kinetic parameters of tumor and limiting normal tissue that must be exploited. In human bone marrow, the most common dose-limiting tissue, cell cycle time, and phase duration are poorly defined but tend to be considerably shorter than the mean values among heterogeneous tumors (tables 2 and 4). If animal experiments are to suggest good schedules in man, they must be performed in animals where kinetics of tumor and bone marrow bear a similar relationship to that in man, and they must study drug effects on bone marrow as well as tumor. Until optimal schedules can be predicted in mice from good information about

kinetics of tumor and bone marrow, it is naive to expect that a rather scanty knowledge of cell kinetics in man will allow prediction of an optimal schedule.

Scheduling in Man

Availability of drugs with intrinsic activity for the tumor is the main determinant of therapeutic effectiveness. If active drugs have greater toxicity for cycling cells and/or preferential toxicity at one phase of the cell cycle, then scheduling can probably influence the TI, as in animals.

Prediction of a precise schedule for a cycle-specific drug would require detailed knowledge of phase duration and its variability in tumor and normal tissue, of lethal activity and cycle progression delay caused by the drug, of clearance of drug, and of differences in pharmacologic interaction with tumor and normal tissue. This degree of complexity renders such predictions valueless.

A general knowledge of cell kinetics in man is, however, of value in establishing some broad guidelines for treatment, or in explaining the success of some empirically derived schedules.

GF is often low in human tumors, so that drugs with cycle specificity are used logically in short courses of treatment, permitting noncycling and therefore resistant cells to re-enter cycle between courses (4,5). The mean cell cycle time of human tumors is often 2–4 days, much shorter than the volume doubling time. Even allowing for considerable heterogeneity, most cycling tumor cells will pass through a drug-sensitive phase within 5–7 days; courses of treatment with phase-specific drugs should probably be no longer, or there will be increased toxicity with little further effect on the tumor. Another observation of potential benefit is the shorter mean duration of S phase in granulocyte precursors of human bone marrow compared with that in most tumors. Active drugs with S-phase specificity might be scheduled with a dose interval long enough to allow normal tissue cells (but not tumor cells) to complete DNA synthesis between periods when the drug is available at toxic levels. While the complexity of the drug-tumor-host interaction makes prediction of this interval impossible, it might be sought in clinical trials comparing different dose intervals.

Scheduling of Single Drugs

Most clinical schedules have been derived empirically and allow a combination of some tumor activity, acceptable normal tissue tolerance, and convenience to patient and physician. Different schedules have been compared objectively for a few drugs; data for the S-phase active drugs cytosine arabinoside and methotrexate offer some insight into the relationship between TI, scheduling, and cell kinetics.

Cytosine arabinoside leads to increasing marrow toxicity with increasing dose if infused for periods of ≥ 48 hours into patients with solid tumors. Daily injections or infusions limited to periods of ≤ 24 hours produced increased toxicity up to a threshold dose, and then no further toxicity with increasing dose (147,148). These results imply relative protection of bone marrow with short infusions or daily injections, presumably because some cells do not enter the sensitive S phase during the period of drug availability. Currently, cytosine arabinoside is used mainly for induction of remission in adult acute leukemia. A study of 159 evaluable patients by the Acute Leukemia Group B found no significant difference in tumor response or bone marrow toxicity when the drug was given as a 7-day course by continuous infusion or by seven daily infusions over 1, 4, or 12 hours (149). Another study of 41 patients found a slight but nonsignificant advantage to continuous infusion of the drug compared with daily injections (150). Remission induction usually involves production of an aplastic marrow with patient survival depending on rigorous supportive therapy; the optimal drug schedule is that which most effectively eradicates leukemic cells and is less dependent on protection of normal tissue.

Twice weekly injections of 30 mg/m^2 of methotrexate were superior to oral daily doses of 3 mg/m^2 in maintaining remission of childhood leukemia (151). The median duration of CR was 349 days with twice weekly treatment, but only 63 days with oral treatment. Toxicity to bone marrow and oral mucosa was similar with the two schedules; the improved TI was probably due to increased tolerance to higher dosage when there was sufficient time between dose intervals to allow cells in normal tissue to complete DNA synthesis and be spared. Correlation between increased toxicity of methotrexate and a short interval between doses was also demonstrated by comparing the toxicity of sequential 5-day courses using the same total dose of 50 mg divided into daily doses of 10 mg or doses of 2.5 mg every 6 hours; injections every 6 hours were much more toxic to bone marrow and gastrointestinal mucosa, and probably all cells entering S phase were killed (152).

Scheduling of Drugs in Combination

Measurement of tumor LI (or proportion of cells in DNA synthesis by FMF) during a course of

chemotherapy has been proposed as a guide to scheduling. Examples include the partial synchrony that has been observed in human melanoma after bleomycin infusion (153), and the correlation of favorable response with an increase in LI after initial cyclophosphamide therapy in bronchogenic carcinoma and in children with neuroblastoma (154,155). For acute leukemia, LI is usually maximal at 48–72 hours after one or more doses of cytosine arabinoside (156–159), and administration of cytosine arabinoside and methotrexate separated by a 48-hour interval has led to longer (but not more frequent) remissions in childhood leukemia than simultaneous administration (158,159). A very high rate of bone marrow remission (84%) was also obtained for 64 previously treated children with acute lymphoblastic leukemia in relapse when vincristine was given 48 hours after cytosine arabinoside in a four-drug combination. However, the remission rate was not significantly less (75%) for 63 patients treated with the three-drug combination (cytosine arabinoside was omitted) (160), and this trial illustrates the danger of attributing favorable results to improvement of scheduling if there is no control group.

Measurement of LI or other parameters during a course of chemotherapy has several limitations. Efficient scheduling requires information about survival of *clonogenic* tumor cells, but the results may be dominated by doomed cells, ie, cells that have been sterilized but which show no morphologic changes recognizable by the light microscope or microfluorimeter, and which may continue to traverse one or more abortive cell cycles before their ultimate lysis. Thus, if a drug leads to the death of 90% of the cells, any synchrony of the surviving 10% may be completely masked prior to lysis of the sterilized cells. Also, most investigators have studied proliferative changes only in the tumor, and concurrent changes in critical normal tissue are usually ignored. In man, rapidly proliferating bone marrow precursors might be more easily synchronized than the heterogeneous slower growing tumor, so that synchrony could be most beneficial if the dose interval is adjusted to protect bone marrow, rather than to maximize antitumor activity. This has rarely been attempted.

LI or FMF probably indicates proliferation of surviving tumor cells if it is measured 2–3 weeks after the last course of chemotherapy, and may then indicate changes in tumor GF. In untreated multiple myeloma, values of LI are commonly in the range of 1%–5% (4,5,67). Treatment, usually with melphalan or cyclophosphamide plus prednisone, may lead to long partial remissions in which the cell number (as measured by production of M protein) decreases to a plateau level. During this partial remission, values of LI may increase sometimes to values as high as 40%–45% (4,5), although high values of LI are not often sustained. Cycle-specific agents have been used to treat a few patients with myeloma at a time when a high value of LI has been induced by prior treatment with alkylating agents. Two patients were found to respond to vincristine with a further reduction in cell number, but it is not known whether this drug increased survival (5). In general, cycle-specific drugs have not proven to be of any benefit in myeloma; the scheduling is only of benefit for drugs with intrinsic antitumor activity.

Scheduling of drugs in combination is extremely complex. New schedules are sometimes introduced with labels such as "synchronizing," "recruiting," or "kinetic" (160–164). Such schedules can lead to a good therapeutic effect, but unless they are compared to treatment with the same drugs administered according to a different time sequence, it is not possible to conclude that synchrony of either tumor or normal tissue is a factor in their success. Additive effects of drugs against the tumor with less than additive toxicity lead to improvement in TI by combination chemotherapy. Experiments in animals have demonstrated that scheduling has the potential to increase differential killing of tumor and normal cells. At present, however, I know of no clinical schedule using combinations of drugs which has taken advantage of the differences in cell kinetics of tumor and normal tissue and which has been shown to be superior to an empirically devised schedule in an adequate sample of patients.

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Acute Myelogenous Leukemia in Patients Receiving Chlorambucil as Long-Term Adjuvant Chemotherapy for Stage II Breast Cancer [1,2]

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SUMMARY

Thirteen consecutive stage II breast cancer patients were treated with long-term adjuvant chemotherapy using chlorambucil. At least three of these patients developed acute myelogenous leukemia (AML). All three patients (and possibly a fourth) who developed AML were postmenopausal, received continuous chlorambucil for ≥ 4 years, had acute red cell anemia at the time of treatment, and had a wbc count in the range of 2700–7700/mm³. After the chlorambucil was discontinued, the wbc count began to slowly rise and the patient developed clinical AML. In all three patients, the diagnosis of AML was established by pathologists on the basis of bone marrow biopsy, aspirate, and peripheral smears. Each of these was subsequently reviewed by the hematologist who treated the patients for AML. Patients who have breast cancer (or any other solid tumor malignancy) are at risk to develop a second malignancy. However, an increasing number of reports are appearing suggesting more than just a casual relationship between leukemia and the use of alkylating agents. This may be related to the dose and duration of therapy with these agents.

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It has been previously reported that acute myelogenous leukemia (AML) has developed in patients receiving chlorambucil. These reports were usually about patients with multiple myeloma or chronic diseases, such as rheumatoid arthritis (1–5). In these patients, chlorambucil treatment was usually short-term, and although the subsequent development of AML may have been related to the chlorambucil, some investigators have stressed that patients with these diseases have a higher than "normal" incidence of AML. Two cases of AML have been reported in patients with metastatic breast cancer. Both of these patients received radiation therapy and multiple-drug cancer chemotherapy (6).

This paper reports the occurrence of AML in three patients while actively receiving long-term adjuvant breast cancer chemotherapy using chlorambucil.

MATERIALS AND METHODS

In 1965, adjuvant breast cancer chemotherapy was begun at the Pennsylvania Hospital. A single agent, chlorambucil, was selected because of the ease of patient management. It could be given orally, usually without nausea, vomiting, or alopecia. The leukopenia was gradual and not associated with the toxic effects of acute neutropenia.

Chlorambucil was started at a dose of 0.2 mg/kg/day and was continued until the wbc count was in the range of 3000/mm³ minimum. Chlorambucil was continued with a daily maintenance dose of 0.03–0.1 mg/kg/day. It was initially planned to continue the chlorambucil indefinitely, or until the patient developed a recurrence of her breast cancer.

Thirteen consecutive women, ages 42–74 years, who had undergone a mastectomy and axillary node dissection were entered in this study (table 1). All of these patients demonstrated metastasis to the axil-

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[4]The technical and manuscript assistance of Ellen Marcovitz is gratefully acknowledged and appreciated.

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TABLE 1.—Adjuvant chemotherapy using chlorambucil in stage II patients with breast cancer

Patient No./age	Postoperative radiation therapy	Duration of daily chlorambucil therapy (yrs)	Development of other solid tumors	Development of AML	Development of metastasis
1/49	Yes	6	No	Yes	No
2/52	Yes	5	No	Yes	No
3/64	No	4	No	Yes	No
4/44	No	5	No	? Probably	No
5/42	Yes	1½	No	No	Yes, died
6/74	No	1	No	No	Yes, died
7/69	No	5	Yes (colon, while receiving chlorambucil)	No	No
8/57	Yes	2½	No	No	Yes, died
9/53	Yes	6	No	No	Yes, died
10/49	No	7½	No	No	Alive and well, no evidence of disease
11/45	No	7	No	No	Alive and well, no evidence of disease
12/43	Yes	6½	Yes (opposite breast, 8 mos after discontinuing chlorambucil)	No	Alive and well, no evidence of disease
13/43	Yes	6	No	No	Alive and well, no evidence of disease

lary nodes and were clinically free of distant metastasis. Seven of these patients also received postoperative radiation therapy to the axillary, supraclavicular, and mediastinal lymph node areas. During the study, patients had cbc, differential, and platelet counts as well as liver and renal function chemistries, chest roentgenogram, skeletal survey, and liver scan. All 13 patients received the adjuvant chemotherapy and were followed by one physician.

Initially, patients were seen weekly with cbc count determinations. After the patient was placed on maintenance chemotherapy, treatment visits were at 3–4-week intervals. Repeat chemistries and roentgenograms were performed periodically. After all 13 patients had been entered, a multiple-drug regimen was selected and continued until the start of the National Cooperative Group Adjuvant Protocols (which used melphalan).

RESULTS

All patients tolerated the chlorambucil extremely well. No treatment was discontinued because of any side effects or toxicity, and all patients were easily placed on maintenance therapy.

Four patients developed metastasis and eventually died from breast cancer. Three patients developed AML. A fourth patient may have had AML; however, she died from an acute myocardial infarction prior to the diagnosis of AML. One patient developed a colon cancer while receiving chlorambucil. Another patient developed a contralateral breast cancer after discontinuing chlorambucil. She underwent a modified radical mastectomy and remains clinically free of disease. The remaining patients remain free of any metastatic disease. All patients have had their chlorambucil terminated and are being closely observed.

CASE REPORTS

The following is a brief discussion of the 13 treated patients. The age listed is the patient's age at the time of mastectomy.

Patient 1.—Patient 1 was a 49-year-old woman with blood-type B positive who received postoperative radiation therapy. She received continuous daily chlorambucil for a 6-year period, during which time her wbc count remained under satisfactory control. After that period, she experienced a drop in hemoglobin from 11.8 to 8.8 g in a 5-week period; her wbc count was $5.5 \times 10^3/mm^3$ at that time. An upper and lower gastrointestinal series was done to determine if she had a bleeding lesion; none was

found. She was given iron orally with no increase in hemoglobin. The chlorambucil was discontinued and a search for the cause of the anemia was continued. Serum folate and B12 levels were normal and iron studies were obtained. The patient's wbc count began to rise. A bone marrow biopsy and aspirate were obtained and the diagnosis of AML was established; the AML was refractory to treatment. There was no evidence of local or distal recurrence of the breast cancer.

Patient 2.—Patient 2 was a 52-year-old woman with blood-type O positive who received postoperative radiation therapy. She received continuous daily chlorambucil for a 5-year period. Over an 8-week period, she had a gradual drop in hemoglobin from 13.6 g (wbc count 5.6 × 10^3/mm^3) to 10.8 g; 4 weeks later the hemoglobin dropped to 8.0 g and then to 7.0 g. She underwent a similar anemia work-up which resulted in a bone marrow biopsy and aspirate diagnosis of AML which also proved to be refractory to treatment. Her peripheral wbc count rapidly rose to 150,000/mm^3 and her platelet count dropped to 20,000/mm^3. There was no evidence of local or distal recurrence of the breast cancer.

Patient 3.—Patient 3 was a 64-year-old woman with blood-type A positive who received no postoperative radiation therapy. She did receive continuous daily chlorambucil over 4 years. During a 12-week period her hemoglobin dropped from 13 to 8.6 g and her wbc count dropped from 2.7 to 1.8 × 10^3/mm^3. She underwent a similar anemia work-up and chlorambucil was discontinued. A bone marrow biopsy and aspirate established the diagnosis. After the discontinuance of chlorambucil, her wbc count promptly rose and her peripheral blood count demonstrated all of the features of AML; the patient then had a refractory clinical course of AML. There was no evidence of recurrence of the breast cancer.

Patient 4.—Patient 4 was a 44-year-old woman who received no postoperative radiation therapy. She received daily chlorambucil continuously for 5 years. Over a 4-month period, there was a gradual decrease in hemoglobin from 11.7 g (wbc count 7.7 × 10^3/mm^3) to 9.8 g; 2 weeks later the hemoglobin dropped to 8.3 g and the following week to 7.7 g. The chlorambucil was discontinued and a rapid anemia work-up was begun. The day prior to her scheduled bone marrow biopsy, she experienced severe chest pain and died in the emergency ward of another institution. An autopsy was performed; the cause of death was an acute myocardial infarction. There was no evidence of local or distal recurrence of the breast cancer. There were no autopsy findings to account for the anemia. A bone marrow examination was not performed by the pathologist because "it is not part of their routine and they find it unrewarding." This patient was suspected of having AML although the diagnosis was not established.

Patient 5.—Patient 5 was a 42-year-old woman with 24 positive lymph nodes who received postoperative radiation therapy and continuous daily chlorambucil for 18 months. She developed distant metastasis and died from diffuse metastases.

Patient 6.—Patient 6 was a 74-year-old woman who received no radiation therapy. She received continuous daily chlorambucil for 12 months and died from metastatic disease.

Patient 7.—Patient 7 was a 69-year-old woman who received no radiation therapy. She received continuous daily chlorambucil for 5 years with no clinical evidence of local or distal recurrence of the breast cancer. She did develop a sigmoid colon cancer which has been resected and the chlorambucil was discontinued. At this time she remains free of any metastatic disease.

Patient 8.—Patient 8 was a 57-year-old woman who received postoperative radiation therapy. She received continuous daily chlorambucil for 2 1/2 years, and then developed fatal diffuse metastases.

Patient 9.—Patient 9 was a 53-year-old woman who received postoperative radiation therapy and chlorambucil daily for 6 years. She subsequently died from metastatic breast cancer.

Patient 10.—Patient 10 was a 49-year-old woman who received no radiation therapy. She received continuous daily chlorambucil for 7 1/2 years at which time it was discontinued. She presently is alive and free of any local or distal recurrent breast cancer.

Patient 11.—Patient 11 was a 45-year-old woman who received no radiation therapy. She received continuous daily chlorambucil for 7 years at which time it was discontinued. She is presently alive and free of any local or distal recurrent breast cancer.

Patient 12.—Patient 12 was a 43-year-old woman who received postoperative radiation therapy. She received continuous daily chlorambucil for 6 1/2 years and was free of any local or distal recurrence. In < 1 year of discontinuing the chlorambucil, a 1-cm nodule became palpable in the remaining breast. It was malignant and a mastectomy was performed. She remains alive and clinically free of any metastasis.

Patient 13.—Patient 13 was a 43-year-old woman who received postoperative radiation therapy. She received chlorambucil daily for 6 years, at which time it was discontinued. She remains clinically free of all metastasis.

DISCUSSION

Rosner et al (7) have recently completed a study of breast cancer and acute leukemia which included

an extensive review of the literature. This excellent review included 13 previously reported breast cancer patients who subsequently developed chronic myelocytic leukemia and added three new cases of chronic myelocytic leukemia. The review included 54 previously reported patients and included 24 new cases of acute leukemia associated with patients who at some time have had breast cancer prior to the development of leukemia. All of these reported leukemias were acute myelocytic except for four cases of acute lymphoblastic.

Of the patients reported by Rosner et al, seven subsequently developed acute leukemia and did not receive any postoperative therapy. The largest group of patients received postoperative radiation therapy. Combination chemotherapy was administered to eight patients with advanced breast cancer. Of these patients, several also received postoperative radiation therapy.

Of this group reported by Rosner et al, the mean interval from the diagnosis of breast cancer to the diagnosis of acute leukemia was 6.9 years. Except for the patients reported in this paper, there did not appear to be any previously reported acute leukemias in breast cancer patients who received adjuvant chemotherapy.

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Protection Against Chemotherapy Toxicity by Iv Hyperalimentation [1,2]

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SUMMARY

A prospective randomized trial was conducted comparing the addition of iv hyperalimentation (IVH) to *Corynebacterium parvum*, isophosphamide, and adriamycin (CIA) chemoimmunotherapy in 26 patients with extensive squamous cell lung cancer. Thirteen patients were entered in each treatment arm of the study and IVH was administered before and after the first course of CIA for a total of 31 days. The major dose-limiting toxic effect of CIA was leukopenia. Less myelosuppression was observed for the patients receiving IVH. The difference in the lowest recorded leukocyte and neutrophil counts between the two groups was significant ($P = 0.03$ and 0.01, respectively). Also, a significant decrease ($P = 0.06$) in nausea and vomiting associated with chemotherapy administration was found for the IVH group. The differences in toxic effects between each group were not maintained over subsequent courses of therapy when both groups received CIA alone. The prevention of the toxic effects of chemotherapy by IVH suggests a means of giving higher chemotherapy doses with the intent of increasing tumor response and patient survival.

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Weight loss prior to therapy is a major adverse prognostic factor for lung cancer patients (1). At our institution, non-oat cell lung cancer patients treated with *Corynebacterium parvum*, isophosphamide, and adriamycin (CIA) chemoimmunotherapy were found to have a significantly poorer response and survival when they had experienced a pretherapy weight loss of > 6% (2). In order to test the hypothesis that nutritional replenishment may increase both response and tolerance to therapy, the addition of iv hyperalimentation (IVH) to the therapeutic regimen was studied.

PATIENTS AND METHODS

A prospective randomized trial was conducted in patients with extensive histologically proven squamous cell lung cancer. Extensive disease was defined according to the Veterans' Administration Lung Cancer Study Group criteria (3,4) as disease which was spread beyond one hemithorax or which was not encompassable within radiotherapy portals of a single hemithorax. Consecutive patients were randomized to receive either CIA plus IVH or CIA alone. Randomization was carried out according to pretherapy weight loss, performance status, and *C. parvum* schedule. All patients who met the above criteria and who were prepared to be hospitalized for at least 5 weeks were accepted. Signed, informed consent explaining the therapy and randomization was obtained prior to randomization according to institutional policy.

IVH was administered for 10 days prior to the first course of CIA and continued up to the start of the second course of therapy for a total of 31 days.

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Patients randomized to receive IVH were admitted to the hospital but were encouraged to maintain full mobility. Patients not receiving IVH had the start of their chemotherapy delayed for 10 days so that they were comparable to the IVH patients who received nutritional replenishment for 10 days prior to their first course of chemotherapy. IVH was given according to the method described by Copeland and Dudrick (5). Infusion was via a central venous catheter inserted percutaneously into the middle of the superior vena cava via the subclavian vein. The infusion rate was increased over the first 3 days in order to supply a minimum of 35 kcal/kg of patient body weight. All precautions recommended for the safe management of IVH were rigidly adhered to (5).

The chemotherapy which was administered in 3-week cycles consisted of isophosphamide given at a dose of 1000 mg/m² in 200 ml of 5% dextrose solution over a 2-hour period daily for 5 days and adriamycin given at a dose of 60 mg/m² in 50 ml of 5% dextrose solution by rapid iv infusion on Day 1 of each course of isophosphamide. *C. parvum* immunotherapy was given sc at a dose of 2 mg/m² according to two randomized schedules. The first was an intensive *C. parvum* schedule consisting of doses administered daily for 10 consecutive days prior to the first course of chemotherapy, on Days 7–16 of the first and second courses, and on Days 7, 12, and 17 of subsequent courses. The second was a nonintensive *C. parvum* schedule consisting of doses given on Days 7, 12, and 17 of each course of chemotherapy.

Evaluation of measurable disease by a full physical examination and chest roentgenogram was also documented before each of the first three courses of chemotherapy and then at 6–12-week intervals. Criteria for response were defined as follows. A complete remission required the disappearance of all clinical, radiologic, and laboratory evidence of tumors. A partial remission was defined as ≥ 50% decrease in the sum of the products of two perpendicular diameters of all measured lesions without simultaneous increase in the size of any lesions or the appearance of any new lesion. Stable disease was defined as a < 50% decrease in the size of measured lesions or an increase of < 25% for a period of at least 12 weeks without the appearance of any new lesions. Progressive disease was defined as an increase of ≥ 25% in the size of any measured lesion or the development of any new lesions.

To assess nutritional status, patients were weighed and anthropometric measurements were recorded before each course of chemotherapy. Weights were expressed as a percentage of the patient's stated normal weight while anthropometric measurements were assessed and expressed as a percentage of a standard value determined for each patient according to the methods described by Jelliffe (6) and Blackburn et al (7). During the first course of therapy, daily oral nutritional intake for both groups was determined by a 24-hour recall method (8), while the nutritional content of the IVH fluid was directly calculated. The nutritional intake was expressed as a percentage of the recommended daily allowance for each patient which was calculated from the patient's pretherapy weight (9). The Karnofsky scale was used to determine performance status (10). Patients had bone marrow examinations to assess cellularity prior to therapy. Cbc, differential, and platelet counts were drawn at least twice weekly during therapy. SMA-12 blood chemistry profiles were drawn before each course of therapy to assess renal and liver function. Nausea and vomiting was graded according to duration: grade I was arbitrarily defined as nausea and vomiting lasting up to 12 hours; grade II, nausea and vomiting lasting from 12 to 24 hours; and grade III, nausea and vomiting lasting for > 24 hours. All other side effects associated with the administration of treatment were recorded and analyzed.

TABLE 1.—Patient characteristics of the 2 randomized groups

Characteristic	No. of patients receiving— IVH	No IVH
Pretherapy weight loss		
< 6%	4	5
≥ 6%	9	8
Pretherapy performance status		
> 70%	6	6
≤ 70%	7	7
Pretherapy skin reaction to dermatophytin		
≥ 10 mm	4	8
< 10 mm	9	5
C. parvum schedule		
Intensive	6	7
Nonintensive	7	6

Prior to initiating any treatment regimen, immunocompetence was evaluated by delayed hypersensitivity skin testing to the recall antigen dermatophytin according to the previously described technique (11). Dermatophytin was chosen because previous results utilizing multiple skin test antigens had indicated that reactivity to dermatophytin at the 10-mm induration level was the most predictable for survival in patients with non-oat cell carcinoma receiving the same chemoimmunotherapy (2).

TABLE 2.—Total nutritional intake from all sources during 1st course of chemotherapy

	Average daily intake (% RDA based on initial weight)* for treatment group—				
	IVH		Non-IVH		
	Median	Range	Median	Range	*P* value
Kcal	170	120–238	81	57–125	<0.01
Nitrogen	270	167–320	133	102–161	<0.01

*RDA = recommended daily allowance.

TABLE 3.—Relation of nutritional parameters to administration of IVH (changes on 1st 2 courses of chemotherapy; patients were randomized to receive IVH during 1st course of chemoimmunotherapy only)

Parameter	IVH group		Non-IVH group		*P* value
	Median	Range	Median	Range	
1st course					
Weight change (% of stated normal weight)	+7	+1 to +11	–1	–9 to +3	< 0.01
Arm muscle circumference change (% of standard)	+6	–2 to +13	–4	–8 to +1	< 0.01
Triceps skin-fold change (% of standard)	–2	–11 to +28	–6	–26 to +29	NS*
2nd Course					
Weight change (% of stated normal weight)	0	–7 to +4	+1	–7 to +7	NS
Arm muscle circumference change (% of standard)	+1	–11 to +3	–5	–8 to 0	NS
Triceps skin-fold change (% of standard)	–8	–16 to +16	–5	–22 to +12	NS

*NS = not significant.

RESULTS

Patient Characteristics

Twenty-six patients were entered into the study. Thirteen patients were randomized to receive CIA and IVH and 13 were to receive CIA alone. Weight loss prior to therapy, performance status, and schedule of *C. parvum* administration were evenly distributed between each arm of the study. Immunocompetence, as measured by dermatophytin skin reactivity, was unevenly distributed and favored the patients not receiving IVH (table 1).

The average daily calories from all sources delivered by the IVH ranged from 2407 to 3818 kcal (median, 3037 kcal). The average daily amount of nitrogen delivered ranged from 13.4 to 20.4 g (median, 14.3 g). Table 2 shows a comparison of the average daily nutritional intakes from all sources for the IVH and non-IVH groups. Patients in the IVH group had more than twice the total calorie and nitrogen intake of the non-IVH group. The effect of IVH on patients' weight and anthropometric measurements is shown in table 3. The group of patients receiving IVH experienced a median weight gain of 7% of their pretherapy weight over the first course of therapy compared to a median weight loss of 1% for the non-IVH group. This difference was significant ($P < 0.01$). The observed weight gain was due to a significant increase in lean body mass as indicated by an increase in arm muscle circumference ($P < 0.01$) rather than to increased fat stores as reflected in the triceps skin-fold measurements. The significant increase in

TABLE 4.—Relationship of response to IVH administration

Response type	No. of patients (%)	
	IVH group	Non-IVH group
Partial	4 (31)	1 (7)
Stable disease	8 (62)	8 (62)
Progressive disease	1 (7)	4 (31)

weight and arm muscle circumference in the IVH group compared to the non-IVH group was not maintained during the second course of chemotherapy when IVH had been discontinued.

Response

Measured partial response was seen in four patients receiving IVH and in one patient not receiving IVH (31% versus 8%). The number of patients with stable disease was the same for both groups but four of 13 patients not receiving IVH showed disease progression compared to only one of 13 patients in the IVH group with disease progression (table 4). These differences are not significant (P = 0.32). No meaningful survival results are as yet available.

Chemotherapy Toxicity

Patients in both groups had normal pretherapy peripheral blood cell counts. The pretherapy bone marrow cellularities of both groups were also similar. The IVH group had a median cellularity of 30% (range, 15%–40%) and the non-IVH group also had a median cellularity of 30% (range, 20%–40%). Both groups had peripheral blood cell counts drawn approximately every third day after therapy. The major dose-limiting toxic effect was leukopenia. A significant difference was found in the lowest recorded leukocyte and neutrophil counts between the IVH and non-IVH groups for the first course of therapy (P = 0.03 and 0.01, respectively). No difference in the times of the maximum recorded blood cell count depression or in the blood cell count recovery was detected between the two groups. The differences in lowest recorded blood cell counts were not maintained at a significant level over the second course of chemotherapy when no IVH was given to both groups and, by the time of the third course of therapy, the influence of prior nutritional replenishment on blood cell count depression for the IVH group had completely disappeared (table 5). The difference of *C. parvum* schedule on myelosuppression was also examined over the first course of therapy. The median lowest recorded leukocyte and neutrophil counts were identical for both groups (2.0 and 0.8 × $10^3/\mu l$, respectively), demonstrating that the schedule of immunotherapy did not influence myelosuppression.

Nausea and vomiting was not a major toxic effect with this therapy, but a significant decrease (P = 0.006) in the duration of nausea and vomiting associated with chemotherapy administration was found for the IVH group. Ten of 13 patients in the non-IVH group had persistent nausea and vomiting lasting for > 12 hours compared to two of 13 patients in the IVH group.

IVH Toxicity

Patients tolerated IVH well. No cases of catheter-related sepsis occurred. Two patients required a

TABLE 5.—Effect of IVH on chemotherapy-induced bone marrow toxic effects (the IVH group received only IVH during 1st course of chemoimmunotherapy)

Course No.	Median lowest recorded peripheral blood cell counts × $10^3/\mu l$		
	Wbcs (range)	Polymorphonuclear neutrophils (range)	Platelets (range)
1			
IVH group	2.5 (1.0–4.1)	1.6 (0.3–3.2)	259 (80–416)
Non-IVH group	1.5 (0.7–3.7)	0.4 (0.02–1.2)	185 (50–415)
	(P = 0.03)	(P = 0.01)	(P = NS)*
2			
IVH group	2.3 (0.6–3.0)	1.1 (0.1–3.0)	200 (25–300)
Non-IVH group	1.9 (0.7–3.7)	0.8 (0.2–1.8)	169 (45–299)
	(P = NS)	(P = NS)	(P = NS)
3			
IVH group	1.8 (0.6–4.3)	0.7 (0.2–3.0)	160 (25–270)
Non-IVH group	1.9 (0.9–4.3)	0.7 (0.2–2.8)	158 (45–305)
	(P = NS)	(P = NS)	(P = NS)

*NS = not significant.

catheter change because of occlusion with thrombus.

DISCUSSION

This is the first reported study demonstrating that nutritional support protects patients from chemotherapy-induced myelosuppressive toxicity and suggests a means of giving higher chemotherapy doses. Copeland et al (5), in a retrospective review of 175 patients receiving IVH and a wide variety of chemotherapeutic drugs, did not identify any protective effect of IVH on myelosuppression; however, this study lacked control patients receiving the same chemotherapy and no IVH. Cohen and co-workers (12) have reported that increasing the dose of chemotherapy above the standard dose of the same drugs in patients with oat cell lung cancer resulted in better responses and longer survivals. Whether this is true for other lung cancers has yet to be determined. However, this study has demonstrated the potential for the administration of higher doses of chemotherapy which may result in an increased patient response and survival to many therapies where myelosuppression is the dose-limiting toxicity.

As a caution, one must consider that the differences in drug toxicity may be due to the effect of forced nutritional support on drug metabolism. It is therefore important to determine the direct effect of nutritional support on drug antitumor efficacy. Lanzotti and others (13), after a retrospective analysis of lung cancer patients given IVH, reported that IVH may favorably influence tumor response to chemotherapy. However, a prospective randomized study looking at this question has not previously been reported. In our study, the relative immaturity and small number of patients do not allow us to demonstrate the direct effect of nutritional support on response and survival. Also, it is doubtful whether a meaningful difference in survival could be maintained unless patients are nutritionally supported through subsequent courses of therapy since, as we have shown, the nutritional advantage gained by the IVH patients was soon lost over subsequent courses of therapy when IVH was not given. Even so, the evidence to date suggests that nutritional support may increase rather than decrease chemotherapeutic efficacy.

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Pharmacologic Disposition of Chlorozotocin in Mice [1,2]

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SUMMARY

Chlorozotocin is a water-soluble chloroethylnitrosourea with the cytotoxic group attached to the C2 position of glucose. The distribution of the alkylating and carbamoylating moieties of the chlorozotocin molecule was determined in mice following the ip administration of an LD10 dose: 20 mg/kg. The half-life ($T_{0.5}$) for the plasma disappearance of intact drug was 5 minutes. The plasma disappearance of the ethyl-^{14}C group was biphasic up to 120 minutes after administration; the $T_{0.5}$ of the initial phase was 17.5 minutes and the $T_{0.5}$ of the prolonged second phase was 107 minutes. The disappearance of glucose-^{14}C chlorozotocin followed kinetics similar to the chloroethyl-labeled compound. Fifteen minutes after administration, ethyl-^{14}C drug concentrated maximally in the liver (194 nmols/g of tissue) and the kidney (131 nmols/g of tissue). Uptake into the bone marrow at 60 minutes after ip administration of the ethyl-labeled drug was 6.6 pmols of the ethyl-^{14}C group covalently bound to proteins and nucleic acids per 10^7 nucleated cells. The concentration of ethyl-^{14}C drug in the brain remained at 4 nmols/g of tissue up to 2 hours after administration, reflecting the water-soluble property of this new nitrosourea.

[Cancer Treat Rep 62:1145–1151, 1978]

The nitrosoureas are an important class of antitumor agents demonstrating a broad spectrum of activity in human cancer. However, these drugs frequently produce delayed and cumulative myelotoxicity associated with extended periods of depressed wbc and platelet counts which limits their clinical usefulness (1,2). This is particularly true of the more lipid-soluble nitrosoureas such as BCNU, CCNU, and methyl-CCNU. Previous structure-activity studies have demonstrated that bone marrow toxic effects can be reduced by attaching the cytotoxic group to the C2 position of glucose (3,4). A recently synthesized compound of this class is the water-soluble nitrosourea, chlorozotocin. Chlorozotocin has in vitro and in vivo alkylating activity that is two- to three-fold more than that of CCNU or BCNU and has essentially no carbamoylating activity as shown in table 1 (5). This compound has curative antitumor activity against L1210 leukemia in mice, comparable to BCNU, at doses resulting in little or no myelotoxicity (6). The present studies were initiated to determine the distribution and rate of degradation of chlorozotocin in mice.

MATERIALS AND METHODS

Male BALB/c × DBA/2F (hereafter called $CD2F_1$) mice, weighing 17–25 g, were kindly supplied by Hazleton Laboratories (Vienna, Va), and were maintained on Lablox laboratory chow pellets and water ad libitum. Chlorozotocin, labeled in either the ethyl or the glucose moiety, was kindly supplied by Dr. Harry Wood, Drug Development Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda,

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[5]We wish to thank Ms. Faye Logan for her assistance in the preparation of this manuscript.

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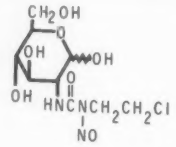
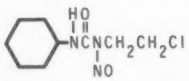
TABLE 1.—Comparison of the in vitro biochemical activities of chlorozotocin and CCNU

Nitrosourea	Structure	Carbamoylating activity (% carbamoylated ^{14}C-lysine)	Alkylating activity (% chlorozotocin)
Chlorozotocin		2	100
CCNU		45	15

Md). The specific activity of ethyl-^{14}C chlorozotocin was 16.1 millicuries/mmol; the specific activity of glucose-^{14}C chlorozotocin was 17.1 millicuries/mmol.

The stability of chlorozotocin was determined in 0.01 M Na citrate buffer (pH 4), 0.25 M sucrose, 0.2 M Na phosphate buffer (pH 7.4), and deionized water. Chlorozotocin was demonstrated to have maximum stability (95% intact drug at 1 hour as measured by ultraviolet absorption at 260 nm) in the citrate buffer. In contrast, chlorozotocin in a 0.25 M sucrose solution demonstrated 65% decomposition by 1 hour and 75% decomposition by 2 hours. Based on these results, the drug was dissolved in 0.01 M citrate buffer, pH 4, and administered ip as a single LD10 dose of 20 mg/kg (64 μmols/kg).[6] This route of administration was selected to be comparable with our previous in vivo toxicologic and therapeutic studies in mice.

The distribution of ethyl-^{14}C chlorozotocin was determined following the ip administration of a single LD10 dose (containing 21.6 microcuries of carbon 14). Aliquots of plasma obtained from the retro-orbital sinus of each of three mice at 2.5, 5, 7.5, 15, 30, 60, 90, and 120 minutes after injection were counted for radioactivity. Heparinized microhematocrit capillary tubes were used to obtain the plasma. In addition, the distribution of ethyl-^{14}C drug in brain, kidney, liver, pancreas, and spleen was determined at 15, 30, 60, 90, and 120 minutes after ip injection. After mice were killed by cervical dislocation, each tissue was collected on ice, weighed, and homogenized in 0.1 M citrate buffer, pH 4, at 4°C (20% wt/vol homogenate). Urine was collected in vials containing citrate buffer. An aliquot of each sample of plasma, tissue homogenate, or urine was added to 10 ml of liquid scintillation fluid (Scintiverse, Fisher Scientific Co, Silver Spring, Md) and counted in a Searle Mark III liquid scintillation spectrometer with automatic quench correction and a counting efficiency of 92% for carbon 14. The half-lives ($T_{0.5}$) were determined from the mean plasma levels of radiolabel using a programmable Hewlett-Packard 97 calculator.

Plasma, tissue homogenates, and urine were chromatographed on thin-layer silica gel plates without fluorescent indicator (Eastman Kodak Co, Rochester, NY) to determine the presence of parent compound and degradation products. The plates were developed overnight using a butanol:acetic acid:water (4:1:2 vol/vol) solvent system (7). Chemically intact chlorozotocin was identified by radioautography of the unchanged parent compound. Radioactivity in the spots was determined quantitatively by cutting out the appropriate areas in strips and placing them in scintillation vials containing 10 ml of Scintiverse. Chloroform:95% ethanol (98:2) and isopropyl alcohol:ammonium hydroxide:water (9:1:1) were two other solvent systems tested for separation of chlorozotocin and its products of degradation. Neither system gave good resolution of the chlorozotocin fractions.

The plasma disappearance of glucose-^{14}C chlorozotocin was determined following the ip administration of a single LD10 dose (containing 22.9 microcuries of carbon 14). Aliquots of plasma obtained from the retro-orbital sinus of each of three mice at 2.5, 5, 7.5, 15, 30, 60, 90, and 120 minutes after administration were counted for radioactivity. In addition, the concentration of glucose-^{14}C drug in brain, kidney, liver, pancreas, and spleen homogenates was determined at 120 minutes.

An aliquot of each ethyl-labeled tissue homogenate at 120 minutes was subjected to 5% trichloroacetic acid (TCA) precipitation for 30 minutes at 0°C and then centrifuged in an Eppendorf Brinkmann Centrifuge 3200; the precipitate was washed twice with 5% TCA. The radioactivity in the precipitate was a measure of the amount of the ethyl-^{14}C group covalently bound to tissue macromolecules.

In vivo studies of drug distribution to the murine bone marrow were carried out using ethyl-^{14}C chlorozotocin and equimolar ethyl-^{14}C CCNU (specific activity, 12.4 millicuries/mmol), a myelotoxic nitrosourea. Either chlorozotocin or CCNU was administered ip at a dose of 64 μmols/kg to three normal mice. Each mouse was killed by cervical dislocation

[6]LD10 dose = dose resulting in the death of 10% of normal mice.

60 minutes after drug administration and bone marrow cells from both femurs and tibias were expressed using phosphate buffered saline (PBS) (144 mM NaCl, 10 mM Na phosphate buffer, pH 7.4). The amount of ethyl-^{14}C group covalently bound to the nucleated marrow cells of each mouse was determined as follows. After contaminating rbcs were osmotically lysed, the nucleated cells were suspended in 0.5 ml of PBS and a cell count was done. One milliliter of 5% TCA was then added to each sample to precipitate proteins and nucleic acids; after 30 minutes at 0°C, the samples were centrifuged in an Eppendorf Brinkmann Centrifuge 3200 and the precipitates were washed twice with 5% TCA. Each precipitate was then suspended in 0.5 ml of PBS and added to a counting vial containing 0.5 ml of NCS Tissue Solubilizer (Amersham Searle, Arlington Heights, Ill); the radioactivity in each sample was then determined.

Blood chemistry tests and tissue histology studies were performed to determine the qualitative organ toxic effects following a single LD10 (20 mg/kg) or LD90 (30 mg/kg) dose of chlorozotocin administered ip; a control group of mice received an ip injection of the citrate vehicle. Three mice from each experimental group were killed daily; blood was collected by cardiac puncture on Days 1–6 and analyzed on an SMAC-23 autoanalyzer. Values obtained from the drug-treated animals were compared with controls. The kidneys and livers from each treatment group were fixed with 10% formalin and stained with hematoxylin and eosin for histologic examination.

RESULTS

Concentration of Chlorozotocin in Plasma

Figure 1 presents the concentration of ethyl-^{14}C chlorozotocin in mouse plasma at serial time points following the ip administration of a single LD10 dose. Ethyl-labeled chlorozotocin attained a maximum plasma concentration of 0.14 mmol/liter within 5 minutes. Quantitatively, this radioactivity in the plasma at 5 minutes represented 9% of the total amount of radioactivity administered ip. The plasma concentration of the ethyl-^{14}C group was 0.08 mmol/liter at 15 minutes and 0.03 mmol/liter at 30 minutes.

The plasma disappearance of the ethyl-^{14}C group was biphasic. The $T_{0.5}$ of the initial phase was 17.5 minutes and the $T_{0.5}$ of the prolonged second phase was 107 minutes. The $T_{0.5}$ calculated for the disappearance of the parent compound was 5 minutes; the percentage of intact drug in the plasma was determined by thin-layer chromatography.

The plasma disappearance of the glucose-^{14}C group followed similar kinetics (fig 1). The $T_{0.5}$ of the initial phase was 22.3 minutes and the $T_{0.5}$ of the prolonged second phase was 180 minutes.

Products of decomposition and possible metabolism of ethyl-^{14}C chlorozotocin in the plasma were separated from intact parent compound by thin-layer chromatography. Radioactivity migrating as a distinct spot with the intact chlorozotocin had an R_f value of 0.56. In addition, a spot representing degradation products was detected at the origin (R_f = 0) and accounted for 15% of the total plasma radioactivity at 15 minutes, 45% at 30 minutes, and 64% at 60 minutes (fig 2).

Thin-layer chromatography of intact radiolabeled chlorozotocin in the citrate vehicle demonstrated that the quantity of impurities was $< 5\%$. This material remained at the origin (R_f = 0) with the products of degradation.

Concentration of Chlorozotocin in Tissues

Figure 3 represents the distribution of the ethyl-^{14}C group per gram of wet weight in brain, kidney, liver, pancreas, and spleen tissue. Fifteen minutes after administration, the drug was concentrated maximally in the liver and the kidney with a peak concentration of 194 and 131 nmols of the ethyl-^{14}C group per gram of tissue, respectively. The concentration of ethyl label in the pancreas gradually increased to reach a maximum of 76 nmols/g of tissue at 120 minutes. Spleen concentration decreased from a maximum of 28 nmols/g at 15 minutes to 18 nmols at 2 hours. In contrast, the maximum concentration in the brain was only 4 nmols/g of tissue; this concentration remained constant up to 2 hours.

In vivo distribution of ethyl-labeled chlorozotocin and CCNU to the bone marrow was determined at 60 minutes after a dose of 64 μmols/kg. The distribution of the ethyl-^{14}C group covalently bound to proteins and nucleic acids per 10^7 nucleated marrow cells was 6.6 ± 1.2 pmols for chlorozotocin and 5.6 ± 1.8 pmols for CCNU. At this equimolar dose, there was no statistically significant difference in the pmols of the ethyl-^{14}C group covalently bound to the nucleated marrow cells.

The concentration of the glucose-^{14}C group of chlorozotocin in tissues was measured at 120 minutes. Table 2 presents the distribution of the glucose-^{14}C group per gram of wet weight in brain, kidney, liver, pancreas, and spleen tissue. The glucose label of chlorozotocin was concentrated maximally in the liver and the kidney with 57 nmols of the glucose-^{14}C group/g of liver and 32 nmols/g of kidney.

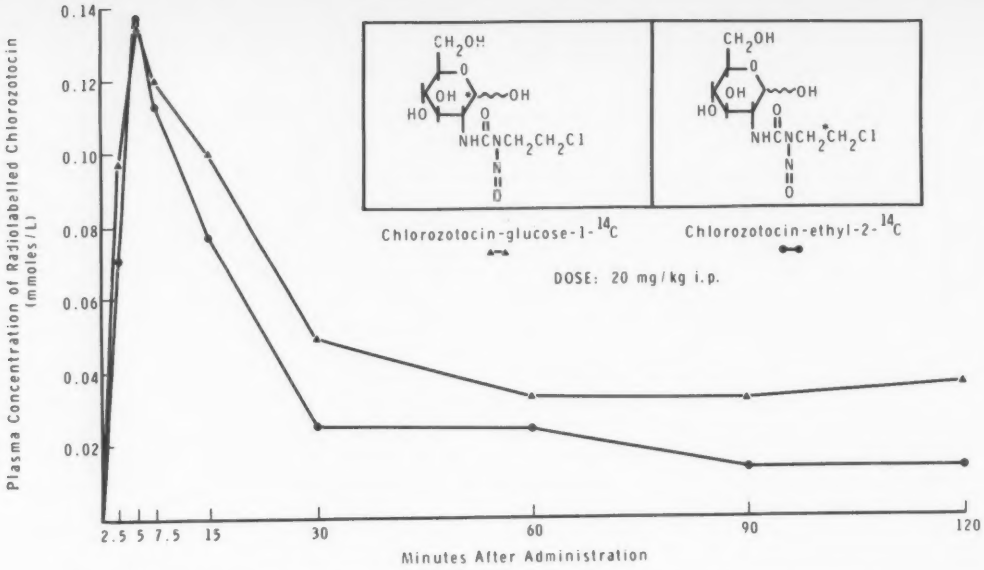


FIGURE 1.—Distribution of the ethyl-^{14}C or glucose-^{14}C group in mouse plasma at serial time points.

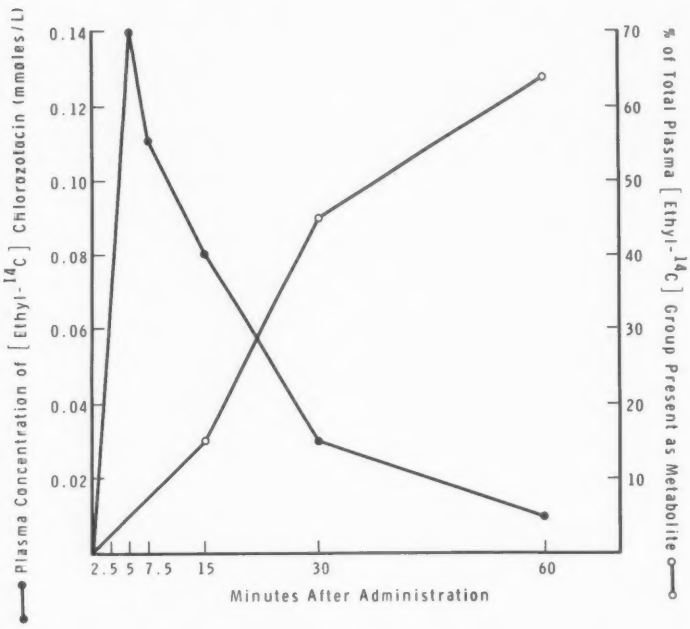


FIGURE 2.—% of total plasma ethyl-^{14}C group present as metabolite.

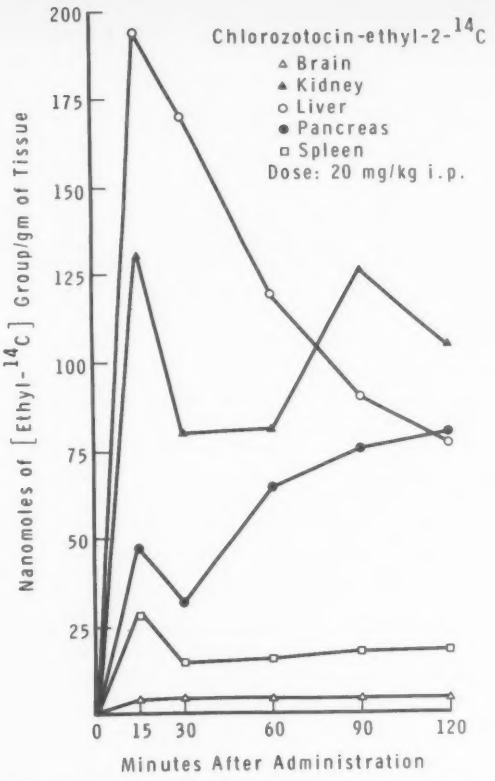


FIGURE 3.—Distribution of the ethyl-^{14}C group in tissues at serial time points.

Results of thin-layer chromatography of the ethyl-labeled tissue homogenates are presented in table 3. By 60 minutes, at least 55% of the radioactivity in the kidney, liver, pancreas, or spleen homogenate was associated with the products of degradation at the origin (R_f = 0). Radioactivity obtained from fractions of brain homogenate was negligible. The in vitro chemical decomposition of chlorozotocin in control tissue homogenates "spiked" with ethyl-labeled drug and maintained at 4°C was

TABLE 2.—Comparison of tissue concentrations of radiolabel 120 mins following ip administration of either glucose-^{14}C or ethyl-^{14}C chlorozotocin (LD10 dose)

Tissue	nmols of glucose-^{14}C group/g of tissue	nmols of ethyl-^{14}C group/g of tissue
Brain	4	4
Kidney	32	105
Liver	57	77
Pancreas	27	79
Spleen	13	18

TABLE 3.—% of total radioactivity in tissues that is present as metabolite 60 mins following ip administration of ethyl-^{14}C chlorozotocin (LD10 dose)

Tissue	% of ethyl-^{14}C group present as metabolite
Kidney	55
Liver	77
Pancreas	73
Spleen	57

determined at serial time points. Analysis by chromatography showed maximum drug decomposition in the liver homogenate; at 15 minutes, 15% of the chlorozotocin had undergone decomposition (R_f = 0) and this value increased to 23% at 60 minutes and 40% at 120 minutes, the maximum time point of the study.

Ethyl-labeled tissue homogenates from the 2-hour time point were precipitated with 5% TCA at 4°C. Table 4 presents the percentage of the ethyl-^{14}C group covalently bound to tissue proteins and nucleic acids. This percentage was highest in the liver (31.3%) and lowest in the pancreas (10.8%).

Concentration of Chlorozotocin in Urine

Thirty minutes following administration of the ethyl-labeled drug, the concentration in urine was 9 mmols/liter; by 60 minutes, the concentration had decreased to 3 mmols/liter.

The products of degradation of ethyl-^{14}C chlorozotocin in urine were chromatographically separated from intact parent compound. At 15 minutes after administration, 66% of the total radioactivity in the urine was in fractions with mobility identical to that of intact chlorozotocin (R_f = 0.56). This value decreased to 41% at 30 minutes and to < 15% at 1 hour.

Chlorozotocin Toxicity

The following changes were observed following treatment with an LD90 ip dose of chlorozotocin (30 mg/kg). On Day 4 after treatment, the average serum creatinine value for the LD90 group was 1.2 mg/dl as compared to 0.3 mg/dl for the control group; this value increased to 2.4 mg/dl on Day 6. On Day 3 after administration, the average BUN value for the LD90 group was 44 mg/dl as compared to 24 mg/dl for the control group; this average BUN value increased to 77 mg/dl on Day 4 and 147 mg/dl on Day 6. The kidney showed pathologic evidence of renal tubular necrosis on Day 5 following treatment

TABLE 4.—% of total radioactivity in tissues that is covalently bound to tissue macromolecules 120 mins following ip administration of ethyl-^{14}C chlorozotocin (LD10 dose)

Tissue	% of ethyl-^{14}C group covalently bound to tissue macromolecules (mean ± SE)
Brain	22.9 ± 2.63
Kidney	17.8 ± 0.58
Liver	31.3 ± 3.95
Pancreas	10.8 ± 1.61
Spleen	19.1 ± 4.58

with the LD90 dose. The liver showed vacuolar changes without necrosis or cellular infiltration following both LD10 and LD90 doses; these changes were maximal at 4 days after drug administration.

DISCUSSION

Under physiologic conditions, chloroethylnitrosoureas decompose to yield chloroethyl carbonium ions or diazonium hydroxides which function as the principal alkylating products (8). In addition, organic isocyanates are formed that carbamoylate the lysine and other residues of proteins (9,10). Chlorozotocin, a water-soluble nitrosourea, has the cytotoxic group attached to the C2 position of glucose. Compared to CCNU, chlorozotocin has more than twice the relative alkylating activity (as measured by the *p*-nitrobenzyl pyridine assay) and essentially no carbamoylating activity, as shown in table 1 (5). The purpose of the present study was to determine the distribution of the alkylating and carbamoylating moieties of the chlorozotocin molecule in mice; this study employed the dose schedule and ip route of administration used in our previous toxicologic and therapeutic studies in this species.

The plasma disappearance of the ethyl-^{14}C group was biphasic with a $T_{0.5}$ of 17.5 minutes for the initial phase and 107 minutes for the prolonged second phase. The $T_{0.5}$ for the disappearance of intact drug was 5 minutes. Chromatography of plasma samples demonstrated that by 60 minutes most of the drug has been degraded and/or metabolized. In previous studies, under conditions of 37°C and PBS, pH 7.4, the chemical $T_{0.5}$ of chlorozotocin was 48 minutes (5). The kinetics of the plasma disappearance of the glucose-^{14}C group were similar to the chloroethyl portion of the molecule.

The liver and the kidney were the principal organs of distribution of both the chloroethyl and glucose portions of the molecule. At 2 hours after administration, the concentration of radiolabel in the kidney, liver, and pancreas was significantly higher for the ethyl as compared to the glucose moiety.

An important but not unexpected result was the low concentration of the ethyl-^{14}C group in the brain. This is an anticipated result in view of the greater water solubility of this analog as compared to other members of this class of compounds, including BCNU and CCNU. This finding is consistent with the demonstration that chlorozotocin has little activity against intracranially implanted L1210.[7] In addition, the results of a recently completed phase I trial in man showed little penetration of either the ethyl or glucose label into the cerebrospinal fluid (11).

Toxicologic studies suggest that the lethal toxic effects in the mouse are renal. This is manifested by an elevation in serum creatinine and BUN levels and evidence of renal tubular necrosis. Two additional correlates are the high concentration of ethyl label in the kidney at 2 hours, of which 18% is covalently bound, and the high concentration of intact chlorozotocin and its degradation products in the urine.

The unique biologic feature of chlorozotocin compared to other nitrosoureas is its ability to produce curative L1210 activity without a reduction in the absolute polymorphonuclear neutrophil count. The differential reduction of chlorozotocin toxicity for the normal murine bone marrow cannot be explained by the in vivo distribution to the marrow. The quantitative covalent binding of the ethyl-^{14}C group of chlorozotocin per 10^7 nucleated cells was equivalent to that observed for CCNU at an equimolar but marrow toxic dose (5).

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Use of Microbiologic and Enzymatic Assays in Studies on the Disposition of 2′-Deoxycoformycin in the Mouse[1,2]

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SUMMARY

Microbiologic and enzymatic assays for 2′-deoxycoformycin (2′-dCF), a potent inhibitor of adenosine deaminase (ADA), were developed, compared, and used in pharmacokinetic studies employing BDF_1 mice. The enzyme assay was based on the degree of inhibition of calf intestinal ADA by 2′-dCF recovered from heat-treated plasma and tissue samples. Inhibition of the enzyme was linear in phosphate buffer in the range of 0.1–0.7 ng. The microbiologic assay was of the agar-disc diffusion type and employed a strain of *Streptococcus faecalis* that was highly susceptible to 2′-dCF inhibition. The assay as measured by zones of inhibition was linear between 0.5 and 10 ng/ml in saline. 2′-dCF was rapidly distributed in the mouse. Within 15 minutes following ip administration of 2′-dCF, the highest initial levels of drug were found in the kidneys, followed by liver and spleen; lower levels of 2′-dCF were found in the thymus, heart, and lung, with negligible drug in the brain. Elimination of 2′-dCF from plasma after 0.25 and 2.5 mg/kg was biphasic with half-lives for the α and β phases of 17 and 64 minutes and 18.5 and 104 minutes, respectively. Urinary excretion accounted for > 90% of 2′-dCF in 2 hours. For an optimal therapeutic dose and schedule (0.05 mg/kg of 2′-dCF plus 80 mg/kg of araA ip every 3 hours × 8 on Days 1, 5, and 9) against P388 and L1210 leukemias in mice, these assays are sufficiently sensitive to measure 2′-dCF in plasma and tissues for at least 2 hours after drug administration. Both assays should prove useful for further studies on the pharmacology of 2′-dCF in other animal species.

[Cancer Treat Rep 62:1153–1159, 1978]

Adenosine deaminase (ADA) has a primary role in the normal catabolic pathway for adenine nucleosides (1). This enzyme catalyzes not only the deamination of adenosine, but also that of araA (9-β-D-arabinofuranosyladenine; vidarabine), an important antiviral and antitumor agent (2–6). Deamination of araA greatly reduces its chemotherapeutic potential as an anticancer agent, and inhibitors of ADA have been sought in an attempt to enhance the limited anticancer activity of araA (7–10). An effective inhibitor of ADA is 2′-deoxycoformycin (2′-dCF), a compound that has recently become available (11,12). Combination chemotherapy with 2′-dCF and araA greatly enhances the antiviral and antitumor activity of araA (13–18).

The inhibition of ADA by erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride has been reported (19,20) to depress human lymphocyte blastogenesis and to enhance the inhibitory effect by adenosine on mitogen-mediated lymphocyte blastogenesis. ADA has recently been reported to be deficient in patients with severe combined immunodeficiency (21–23). Although a direct causal relationship between enzyme deficiency and immunocompetence

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has yet to be established, there is the possibility that ADA inhibitors will prove to be effective immunosuppressants (24,25).

Because of the potential usefulness of 2′-dCF in clinical therapy, there is a need for a simple and reliable quantitative assay for use in measuring this agent in the body fluids and tissues of experimental animals and treated patients. As an approach to this problem, we have developed both a microbiologic and an enzymatic assay for 2′-dCF. The present study involves a comparison of these two assays and their application in determining the pharmacokinetics of 2′-dCF in BDF_1 mice. Preliminary reports of these data have appeared (26,27).

MATERIALS AND METHODS

Materials

Adenosine was obtained from P-L Biochemicals, Inc (Milwaukee, Wis). ADA (Type III) and 2,6-diaminopurine (DAP) were obtained from Sigma Chemical Co (St. Louis, Mo). 2′-dCF (> 94% chemically pure by ultraviolet analysis) was provided by Parke-Davis & Co and by the Drug Development Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

Experimental Protocol

BDF_1 male mice (weight, 25–30 g) in three groups (three mice/group) were given a single ip dose of 2′-dCF dissolved in either phosphate buffer or 0.9% saline. The volume of the injection solution ranged from 0.2 to 0.5 ml/mouse. At various time intervals after injection, the animals were anesthetized, blood was taken by cardiac puncture, and the plasma was obtained by centrifugation. Urine was collected by external catheter or in short-interval experiments directly from the bladder after the animals were killed. The tissues were excised and placed on ice. Tissue weights were recorded, and tissues were homogenized in phosphate buffer or 0.9% saline by use of a Brinkmann polytron homogenizer with a P–10 generating head. Tissue homogenates were used directly for the microbial assay. For the enzyme assay, the homogenates were centrifuged at 28,000 × *g* and the supernatants collected. The tissue pellets were resuspended and washed twice with 1 ml of phosphate buffer, and the supernatants of these washings were added to the original supernatant. Tissue samples were either used immediately after being processed or were stored at –20°C until assayed. 2′-dCF was stable in tissue samples stored at –20°C; repeated freezing and thawing of tissues had no apparent effect on drug assay activity.

Enzyme Assay

The enzymatic method was based on the inhibition of calf intestinal ADA by 2′-dCF extracted from mouse tissue and fluids. ADA was incubated at 25°C with varying amounts of 2′-dCF or sample to be assayed in a microliter polyethylene ladle for 3 minutes (12); further incubation with 2′-dCF did not increase the inhibition of the enzyme. Prior to analysis for 2′-dCF, the samples of urine, plasma, and tissue were heated at 100°C for 6 minutes to inactivate any endogenous ADA that might have been present and disrupt protein binding of 2′-dCF.

A dose-response curve for inhibition of ADA by 2′-dCF was obtained by comparing the rate of deamination by the control enzyme with the rate following addition of 2′-dCF. Serial dilutions of the tissue supernatants, plasma, and urine were made with phosphate buffer until the concentration of unknown 2′-dCF was in a measurable range (0.1–0.7 ng). For each experiment, known concentrations of 2′-dCF were assayed in phosphate buffer and in a control urine, plasma, or tissue sample.

The assay system consisted of 53 μM adenosine in 0.05 M phosphate buffer, pH 7.5, and ADA (0.027 unit/0.02 ml) or enzyme and substrate plus 2′-dCF (0.1–0.7 ng/0.02 ml) in a final volume of 3 ml. The absorbance of the assay system was measured every 30 seconds for 2 minutes on a Beckman DU-2 spectrophotometer at 265 nm. Phosphate buffer (0.05 M, pH 7.5) was used to dilute the ADA and to make serial dilutions of 2′-dCF. All tissue samples and fluids were assayed at least in triplicate.

Microbial Assay

A laboratory strain of *Streptococcus faecalis* that requires purines for growth ($SFpu^-$) was used for the assay of 2′-dCF. The assay medium was that described by Flynn et al (28) without purine and pyrimidine supplements, except for the addition of DAP at a final concentration of 10 μg/ml. Difco agar, when required, was added at a final concentration of 1.5%.

A subculture of $SFpu^-$ was prepared in Difco folic acid assay broth medium and incubated at 37°C for 18–24 hours. The subculture was centrifuged, and the cells were washed twice with 0.9% saline and resuspended in saline to about 3×10^7 colony-forming units/ml. Ten milliliters of the cell suspension was mixed with each liter of agar medium at 50°C, dispensed into 15 × 100-mm disposable plastic Petri dishes (6.0 ml/assay plate), and allowed to

solidify at room temperature. The assay plates were stored at 5°C for up to 24 hours.

Stock solutions of 2′-dCF for standard curves were prepared in saline and serially diluted in saline, mouse urine, plasma, or appropriate tissue homogenates. Paper discs (Schleicher and Schuell; 0.63-cm diameter) were saturated with 0.02 ml of each solution and placed onto the surface of the agar assay plates. Each plate contained three standard dilutions and two dilutions of a test sample. The difference between the high and low standard dilution was fourfold. The plates were incubated for 18 hours at 37°C, at which time zones of growth inhibition were measured. The average of at least three zone diameter measurements was plotted against the $\log_{10}$ of the 2′-dCF concentration. Standard curves were prepared according to the method of least squares. Unknown tissue and urine samples were assayed in triplicate.

RESULTS

Enzyme Assay

A typical standard curve for inhibition of ADA by 2′-dCF is shown in figure 1. The inhibition of ADA was linear from 0.1 to 0.7 ng of 2′-dCF. The coefficient of variation $\left(\frac{SD}{\bar{X}}\right)$ for quadruplicate samples of 2′-dCF was 0.051.

In the biologic samples, an endogenous source of ADA was sometimes present and could have affected the rate of deamination. Heat treatment of samples (100°C for 6 minutes) generally did not affect the recovery of 2′-dCF. Recovery experiments (total No. = 16) utilizing known amounts of 2′-dCF added to most body fluids and tissues gave 91% ± 6.5% recovery. Exceptions were in liver and whole blood, where recoveries of 79% and 84% of the 2′-dCF, respectively, were observed. The average error of reproducibility for duplicate recovery experiments was < 5%.

Microbial Assay

The purine requirement for the growth of SFpu⁻ was found to be satisfied by either adenine, guanine, hypoxanthine, their nucleosides, or DAP. 2′-dCF inhibited the growth of SFpu⁻ only in the presence of adenine, adenosine, or DAP. The strain was highly sensitive to 2′-dCF in the presence of DAP, and for this reason DAP was used as the purine supplement to the assay medium.

A typical dose-response curve to 2′-dCF in saline is presented in figure 2. Linearity of response was obtained between 0.5 and 10 ng/ml although activity

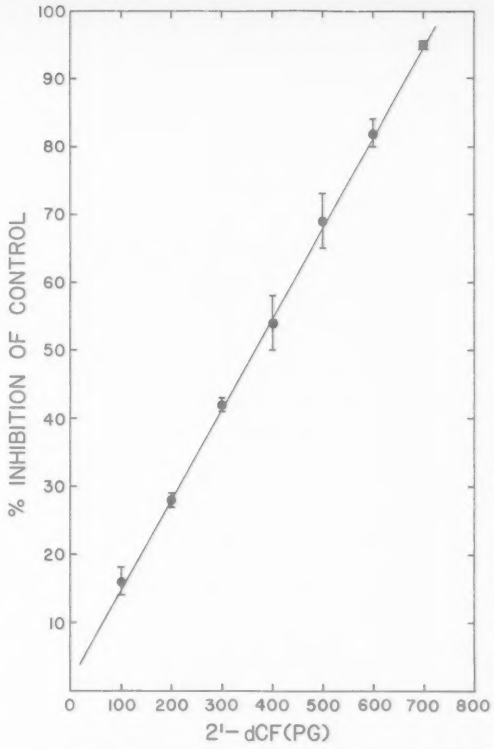

FIGURE 1.—Standard curve for enzymatic assay of 2′-dCF. Each point = mean ± SD of 4 assays.

was detectable at 0.1 ng/ml. Above 10 ng/ml, a linear response was obtained if the square of the diameter of the zone of inhibition was plotted against the $\log_{10}$ of the concentration of 2′-dCF. This was done when dose-response curves were prepared in certain tissue homogenates. The limits of assay sensitivity in various mouse tissues ranged from 1.0 ng/ml in plasma to 20 ng/ml in spleen. The coefficient of variation for duplicate samples of 2′-dCF was calculated to be 0.088.

Animal Studies

The distribution of 2′-dCF in various mouse tissues is presented in table 1. 2′-dCF was rapidly distributed following ip injection. The highest tissue levels occurred within 15 minutes. Higher levels of drug were found in liver, kidney, and spleen than in lung, heart, pancreas, thymus, and brain.

The disappearance of 2′-dCF from mouse plasma is presented in figure 3. Peak plasma levels for 2′-dCF of about 0.24 μg/ml were obtained within 15 minutes after an injection of 0.25 mg/kg. After 2.5

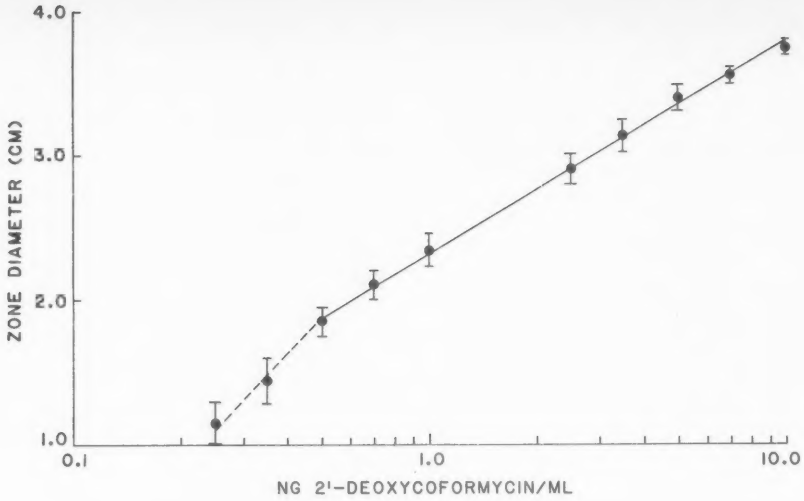

FIGURE 2.—Dose-response of SFpu⁻ to 2'-dCF dissolved in saline. Each point = mean ± SD of 5 determinations.

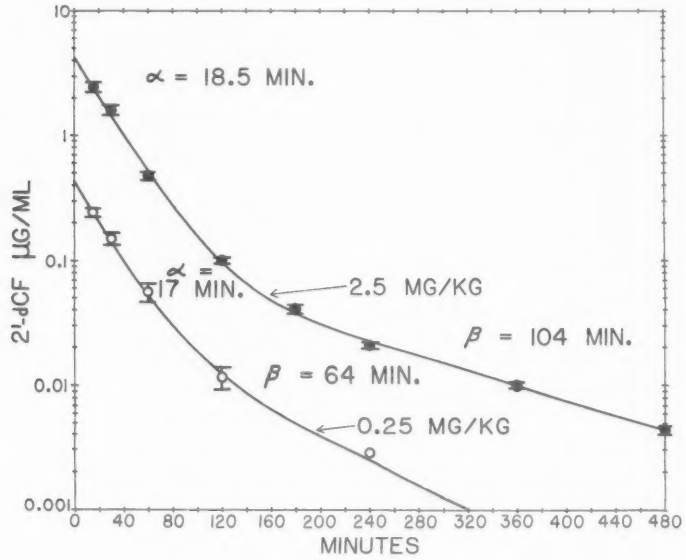

FIGURE 3.—Elimination of 2'-dCF from mouse plasma after ip injections of 0.25 mg/kg (microbial assay) and 2.5 mg/kg (enzymatic assay). Each point = value for pooled plasma from 3 groups (3 mice/group) ± SE.

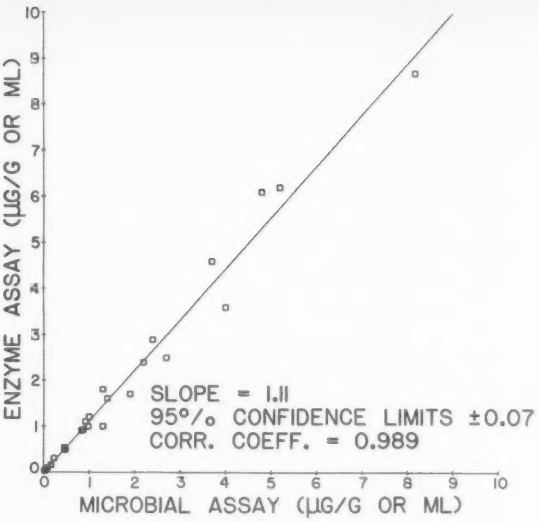

FIGURE 4.—Correlation curve showing comparison of the microbial and enzymatic assays.

mg/kg, the highest plasma levels for 2′-dCF of 2.4 µg/ml also were obtained within 15 minutes. Elimination of 2′-dCF from plasma after 0.25 and 2.5 mg/kg was biphasic, with half-lives for the α and β phases of 17 and 64 minutes and 18.5 and 104 minutes, respectively. The α and β phase half-lives at the two dose levels are not significantly different ($P > 0.05$).

The data on the urinary excretion of 2′-dCF are presented in table 2. Urinary excretion accounted for > 90% of the 2′-dCF within 2 hours.

Separate studies were performed to compare the microbial and enzyme assays. Duplicate tissue samples and body fluids were assayed for 2′-dCF; the data are shown in figure 4. Linear regression analysis gave a slope of 1.11 ± 0.07 (95% confidence limits).

DISCUSSION

The microbial and enzyme assays are highly sensitive and reproducible for use in the measurement of 2′-dCF in animal tissues and fluids. Both assays are sufficiently sensitive to measure 2′-dCF in plasma and tissues for at least 2 hours after administration of 0.05 mg/kg 2′-dCF ip. This dose of 2′-dCF in combination with araA (80 mg/kg, ip) has been found[5] to be effective against P388 and L1210 leukemias in mice on a treatment schedule of every 3 hours × 8 on Days 1, 5, and 9. A comparison of the two assays gives excellent agreement on measurements of duplicate tissue samples and body fluids. Each assay offers certain advantages when compared to the other. Although the microbial assay can be used to analyze more samples at one time than the enzyme assay, the enzyme assay has greater sensitivity for drug detection in certain tissues, such as spleen and brain. Also, for the microbial assay, it is necessary to prepare standard curves of 2′-dCF in each tissue homogenate examined, whereas it does not appear necessary to do this for the enzyme assay. Further, we do not

[5]Annual Progress Report to Division of Cancer Treatment, National Cancer Institute, contract N01-CM-43756, Jan 1977.

TABLE 1.—Levels of 2′-dCF in tissues of BDF$_1$ mice after single ip injections of 0.25 and 2.5 mg/kg*

	2′-dCF: µg/g of tissue							
	15 mins		30 mins		60 mins		120 mins	
Sample†	0.25	2.5	0.25	2.5	0.25	2.5	0.25	2.5
	(Dose in mg/kg)							
Liver‡	0.31 ± 0.02	5.1 ± 1.0	0.19 ± 0.02	3.6 ± 0.26	< 0.06§	1.9 ± 0.72	< 0.06	0.17 ± 0.014
Kidney	0.49 ± 0.06	7.8 ± 1.3	0.31 ± 0.05	3.7 ± 0.49	0.13 ± 0.03	2.2 ± 0.28	< 0.06	0.4 ± 0.10
Lung	0.17 ± 0.04	1.5 ± 0.21	0.15 ± 0.01	1.3 ± 0.28	< 0.10	1.1 ± 0.06	ND	0.19 ± 0.014
Spleen	0.20 ± 0.04	4.6 ± 1.0	0.19 ± 0.01	2.4 ± 0.27	ND	1.2 ± 0.16	ND	0.32 ± 0.012
Brain	< 0.13	0.053 ± 0.010	< 0.13	0.045 ± 0.010	< 0.13	0.035 ± 0.010	< 0.13	0.006 ± 0.001
Heart	ND¶	1.7 ± 0.73	ND	1.2 ± 0.10	ND	1.1 ± 0.010	ND	0.11 ± 0.010
Thymus	ND	1.9 ± 0.56	ND	1.4 ± 0.21	ND	1.0 ± 0.24	ND	0.24 ± 0.070
Pancreas	< 0.13	ND	< 0.13	ND	< 0.13	ND	< 0.13	ND

*Microbiologic (0.25 mg/kg) and enzyme (2.5 mg/kg) assays were performed as described in the Materials and Methods section.
†Samples are pooled from 3 mice/group. Values are from 3 groups ± SD.
‡Values given for 2.5 mg/kg of 2′-dCF are corrected on the basis of 79% recovery.
§Drug concentrations listed after the less than symbol (<) are the limits of assay sensitivity for a particular tissue.
¶ND = not determined.

TABLE 2.—Cumulative urinary excretion of 2′-dCF in BDF_1 mice*

	Dose schedule†	
	0.25 mg/kg, ip	2.5 mg/kg, ip
	% of dose	
Mins	Microbial assay	Enzyme assay
15	ND‡	23 ± 8
30	ND	27 ± 4
60	ND	33 ± 10
120	90 ± 14	93 ± 18
240	ND	102 ± 9
360	95 ± 11	ND

*Enzyme and microbial assays were performed as described in the Materials and Methods section.

†Samples are pooled from 3 mice/group. Values are from 3 groups ± SD.

‡ND = not determined.

believe that the lower recoveries of 2′-dCF from whole blood and liver as compared to other samples limit the use of the above assays. Since the accuracy and reproducibility of our recovery studies are within acceptable limits, appropriate recovery correction factors applicable to such tissues can be applied.

The data from both assays are in agreement with Borondy et al (29) who studied the disposition of radiolabeled 2′-dCF in the rat. These authors found that after an im injection of 2 mg/kg, the drug was rapidly distributed to all body tissues with the highest concentration in the kidneys. Also, they found that > 80% of the drug, mostly unchanged, was excreted in the urine. A preliminary report (30) has appeared on the disposition of 2′-dCF (0.1–1.0 mg/kg iv) in dogs. Using an enzyme assay similar to the one presented here, these investigators found the highest levels of 2′-dCF in lung, spleen, and pancreas, and a half-life in plasma of 75–120 minutes.

Other workers (31) have studied the distribution of ADA in various tissues of the mouse. In comparison to our studies on the disposition of 2′-dCF, these investigators found ADA activity as follows: spleen > lung > liver > kidney > heart > brain. In general, the level of 2′-dCF and the ADA activity in the various tissues of the mouse agree, since we found the highest levels of 2′-dCF in kidney, liver, spleen, heart, and lung.

Due to the potential usefulness of ADA inhibitors in chemotherapy and immunosuppression, the assays described here should prove valuable for further studies on the pharmacokinetics of 2′-dCF and similar inhibitors in other species, including man.

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Prediction of Adriamycin Disposition in Cancer Patients Using a Physiologic, Pharmacokinetic Model [1,2,3,4]

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SUMMARY

A ten-compartment flow-limited pharmacokinetic model scaled from rabbit tissue distribution data was used to predict plasma adriamycin concentrations in 23 patients and adriamycin tissue uptake in nine surgery patients following iv bolus doses of 10–60 mg/m². The predicted concentrations were compared to experimentally determined adriamycin using a specific thin-layer chromatographic fluorescence scanning procedure. The predicted plasma time course for 11 of 16 patients with relatively normal liver and kidney function agreed closely with the observed plasma time course. Deviations in the other five patients were ascribed to possible changes in the profile of metabolite formation and/or fluctuations in biliary clearance. All four patients with elevated serum bilirubin demonstrated significantly higher and more prolonged plasma levels than predicted. The results of two patients with impaired kidney function and one patient with both hepatic and renal involvement were inconclusive. The comparison between predicted and observed tissue concentrations in biopsy samples was varied; however, all were within an order of magnitude. It is concluded that the model depicts adriamycin uptake and distribution reasonably well; however, more needs to be known concerning individual variation in metabolic and biliary excretion rates for this to become more patient-specific. Also, a tumor compartment appears to be an important addition in modifying the model to allow for clinical utility.

[Cancer Treat Rep 62:1161–1171, 1978]

Adriamycin, an anthracycline antibiotic isolated from *Streptomyces peucetius*, has been demonstrated to be effective against a broad spectrum of human neoplasms (1–4). Initial pharmacologic studies (5) and early clinical experience (6,7) have supported the administration of this drug by an intermittent single iv bolus dose. The studies of Benjamin et al (5) demonstrate the need for dose reduction in patients with significantly impaired liver function. Pharmacokinetic studies reporting a relatively long plasma half-life for parent drug and substantial prolonged plasma concentrations of metabolite (8) also support this type of dose schedule. Adriamycin disposition in man following an iv bolus dose was originally described as biphasic (8), which suggested a fairly rapid distribution phase followed

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[4]Adriamycin was supplied by the Developmental Therapeutics Program, DCT, NCI, Bethesda, Md.

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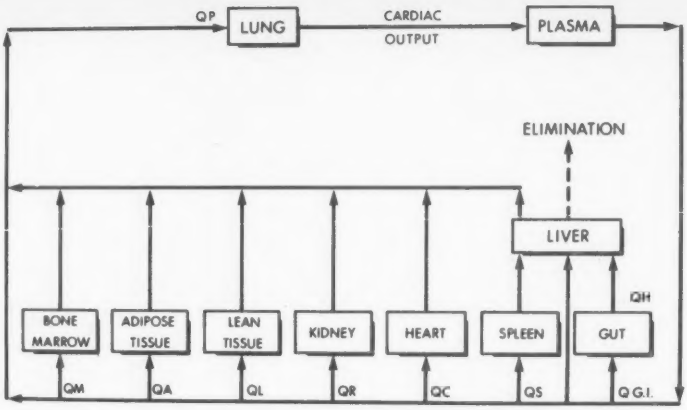

FIGURE 1.—Schematic diagram of the 10-compartment physiologic, pharmacokinetic model developed for adriamycin.

by a log-linear terminal phase with an average half-life of 17 hours. Improvement in assay specificity led to a modification of this kinetic picture which described a triphasic plasma disappearance curve and a long, although quite variable, terminal phase half-life (9,10).

The apparently variable clearance, coupled with the strict limitation in total dose because of potentially life-threatening cardiomyopathy (11), suggests that a more detailed understanding of the kinetics of disposition and elimination of adriamycin is required to allow optimal dose and schedule to be developed on an individual patient basis. In an effort to obtain this detailed description, a Bischoff-Dedrick type multicompartmental flow-limited pharmacokinetic model has been developed in our laboratories (12). This model, which depicts the body as being composed of ten compartments, was developed from rabbit drug distribution data and then scaled to man. The original paper included data for a single patient comparing the model-predicted and observed plasma time course. In order to more fully evaluate the strengths and weaknesses of any model scaled from animals, it is necessary to compare the observed plasma and/or tissue concentrations with those predicted from average model parameters for a variety of different patients. In this paper we present the application of this preliminary model to the prediction of the plasma time course for adriamycin in 23 cancer patients and biopsy tissue concentrations in nine surgery patients following administration of single iv bolus doses ranging from 10 to 60 mg/m^2.

MATERIALS AND METHODS

Model Development

Previously reported information concerning extensive tissue binding of adriamycin (13–15) suggested that a physiologically based model could provide a more useful representation of disposition than a model based solely on plasma concentration data. The approach to developing a physiologic multicompartmental model was similar to that described previously for thiopental (16) and methotrexate (17,18). The blood flow-limited distribution rate model in rabbits and man utilized literature data for blood flows and tissue volumes (table 1). Adriamycin tissue binding was determined experimentally, as were rates of absorption and elimination and plasma protein binding. Experimentally determined disposition parameters for adriamycin in the rabbit are listed in table 2. Ten compartments, namely, plasma, lung, heart, marrow, adipose and lean tissue, kidney, spleen, gut, and liver, were included. The model is schematically represented in figure 1. The mathematic description of the model and the physiologic basis for scaling from rabbit to man have been published in detail previously (12).

For purposes of this preliminary model, clearance of adriamycin from the blood was represented by a total hepatic clearance term which combined hepatic metabolism and biliary excretion. This appears warranted based upon agreement found between adriamycin clearance in rabbits calculated from

TABLE 1.—Physiologic parameters for adriamycin modeling (*from* ref 12)

Parameter	Rabbit*	Man
Body weight (kg)	2.33	70†
Tissue volume (ml)		
Plasma	70	2670
Lungs	17 (3.4)	600
Liver	100 (12)	1700
Kidney	15 (3)	1060
Heart	6 (0.72)	450
Red marrow	47 (7.5)	1400
Adipose tissue	120 (1.2)	7980
Lean tissue	1350 (27)	38,250
Gut	120 (3)	3180
Spleen	1 (0.16)	200
Blood flow (ml/min)		
Plasma	520	5600
Lungs	520	5600
Liver	177	1450
Kidney	80	1240
Heart	16	240
Red marrow	11	120
Adipose tissue	32	200
Lean tissue	155	730
Gut	111	1100
Spleen	9	200

*Values in parentheses = equilibrium blood in tissue.
†1.73 m^2.

TABLE 2.—Disposition parameters of adriamycin in the rabbit (*from* ref 12)

Parameters	
Elimination clearance	70 ml/min (assigned totally to liver compartment)
Plasma protein bound fraction	0.5 (constant)
Intestinal absorption	0
Equilibrium tissue/plasma concentration ratios (± SE)	
Retroperitoneal fat pad (adipose tissue)	19 ± 3.5
Skeletal muscle (lean tissue)	29 ± 3.6
Liver	45 ± 8.3
Small intestine (gut)	51 ± 7.7
Heart	57 ± 5.0
Femur marrow (red marrow)	91 ± 13
Lungs	155 ± 24
Kidney	512 ± 45
Spleen	556 ± 46

total body clearance and hepatic portal vein infusion data which resulted in a loss of 60% in a single pass through the liver. In scaling to man, the product of the hepatic blood flow (1450 ml/70 kg), which was corrected for plasma flow using a hematocrit value of 45%, and the extraction fraction of 0.6 yielded an average total hepatic clearance term of 480 ml/minute for a 70-kg individual. Generally, all previous studies indicated limited urinary excretion of adriamycin with the exception of a recent report by Creasey et al (19). A slight modification from the original model was made with respect to the fraction of drug bound to plasma proteins. Analysis of the original Scatchard plot data indicated that the fraction bound in the therapeutic plasma concentration range was 0.9 rather than 0.5 as originally reported (12). This value was assumed to be a constant.

Patients

Thirty-two patients with solid tumors admitted to the Oncology Service at either John Wesley County Hospital or the Los Angeles County-University of Southern California Medical Center were studied. All patients had received prior chemotherapy or radiation therapy and many were receiving adriamycin as part of a combination chemotherapy protocol. Four patients had elevated serum bilirubin, two had elevated serum creatinine, and one had both kidney and liver function impairment at the time of study. The adriamycin dose ranged from 10 to 60 mg/m^2 depending upon liver function and/or protocol requirements. Adriamycin was administered into a running iv infusion of normal saline or 5% dextrose in water over a 1–2-minute period.

Analysis of Samples

Heparinized venous blood samples were collected before drug administration and serially at specified intervals up to 120 hours after administration. Samples were immediately centrifuged and the resultant plasma was separated and frozen at –20°C until analysis. Biopsy tissue samples were obtained during surgery 1.5–4.0 hours following administration of a bolus iv dose and were also frozen until analysis. Adriamycin in plasma was analyzed using a rapid, highly sensitive thin-layer chromatographic fluorescence scanning technique developed in our laboratories (20). All samples were analyzed in duplicate with the average value used for data analysis. This method is specific for adriamycin and has a lower limit of sensitivity of 2 ng/ml. The standard deviation (SD) for replicate analyses of a 50-ng/ml plasma standard was 3% and the average deviation between duplicates for all patient samples was 3.9%. This procedure also allows reliable quantitation of several adriamycin metabolites with com-

TABLE 3.—Adriamycin plasma time course comparison for 23 patients following an iv bolus dose

Patient No.	Dose (mg/m^2)	Disease	Impaired renal/hepatic function	Mean square error*	Subjective fit†
1	30	Hepatoma	—	0.017	++++
2	40	Bronchogenic carcinoma	—	0.018	++++
3	60	Bronchogenic carcinoma	—	0.029	+++
4	40	Breast carcinoma	—	0.029	+++
5	35	Breast carcinoma	—	0.030	++
6	30	Squamous cell carcinoma	—	0.035	++
7	35	Soft tissue sarcoma	—	0.041	++
8	20	Bronchogenic carcinoma	—	0.046	++
9	40	Bronchogenic carcinoma	—	0.047	+
10	30	Colon carcinoma	—	0.036 (iv)	++
	30	Colon carcinoma	—	0.113 (intra-arterial)	0
11	30	Embryo cell carcinoma	—	0.042	+
12	60	Renal cell carcinoma	—	0.109	0
13	40	Bronchogenic carcinoma	—	0.109	0
14	30	Myeloma	—	0.145	0
15	30	Embryo cell carcinoma	—	0.154	0
16	40	Bronchogenic carcinoma	—	0.192	0
17	10	Bronchogenic carcinoma	Hepatic	0.083	0
18	45	Hepatoma	Hepatic	0.061	0
19	30	Hepatoma	Hepatic	0.071	0
20	15	Hepatoma	Hepatic	0.092	0
21	30	Adenocarcinoma	Renal	0.020	+++
22	60	Renal cell carcinoma	Renal	0.074	0
23	30	Adenocarcinoma	Hepatic/renal	0.422	0

* Mean square error = $\sum_{i=1}^{n} \frac{(\log C_{observed} - \log C_{predicted})^2}{n}$

† Subjective fit scale of 0 to ++++ is in increasing order of goodness-of-fit for a visual estimate.

parable sensitivity. Tissue concentrations of adriamycin were analyzed by the method of Chan and Harris (21).

Model Prediction of Adriamycin Plasma Concentration

Model predictions of adriamycin plasma and tissue concentrations were generated using a digital computer program (PK/MOD, University of Arizona) from blood flows, tissue masses, binding constants, and plasma clearances scaled from average rabbit data to man. A continuous plasma concentration versus time curve was produced for each patient by utilizing individual weights and doses. Integration also provided similar tissue concentration versus time curves simultaneously for the nine other compartments modeled. In addition to these continuous curves, predicted adriamycin concentrations can be obtained for any specific time following drug administration. A typical set of data for an individual patient details predicted concentrations at 15, 30, 45, 60, 90, and 120 minutes and then every hour for as long as desired.

Statistical Treatment

The comparison between predicted and observed adriamycin concentration was made by calculating a mean square error term for six time points for each patient. Since adriamycin plasma concentrations spanned several orders of magnitude, compar-

ative plots were made using semi-logarithmic paper and the mean square error was calculated from differences in log C_p and log C_a where C_p and C_a were the predicted and observed adriamycin concentrations at time, t_x, respectively. Mean square errors of < 0.050 were generally consistent with a reasonable subjective visual fit between predicted and experimental data for an individual patient. Although this was somewhat arbitrarily chosen, the mean square errors calculated are in qualitative agreement with subjective assessment of the goodness-of-fit (table 3).

A second measure of predictive capability was obtained by examining whether the physiologic model predictions lay within ± 1 SD of the classic compartmental nonlinear regression of the observed plasma concentration × time data. The data were fit to a three-compartmental model by computer analysis using the program NONLIN (22). Statistical variation in least-square estimates can be assessed if the following assumptions are made: (*a*) the data include a representative sample of the system; (*b*) the data are statistically independent; (*c*) the data have the same variance (possibly unknown); (*d*) the independent variable (time) is known without error; and (*e*) the uncontrolled error is randomly distributed (23). The 1-SD limits for the nonlinear least-square three-compartment fit of the data were determined as described by Isacoff et al (24). The triexponential decay curve for adriamycin was expressed by:

$$C = \sum_{i=1}^{3} A_i e^{-\alpha_i t} \qquad [1]$$

The statistical error was determined by expressing the function as

$$y = \ln C = \ln\left(\sum_{i=1}^{3} A_i e^{-\alpha_i t}\right) + E \qquad [2]$$

where E is the error and A_i and α_i are constants determined by minimizing the sum of squares (SS) function

$$\sum_{i} [\ln C(t_i) - \ln C_i(t_i)]^2. \qquad [3]$$

The variance of the regression estimate is

$$\text{var}\,(E) = \sigma^2 = \frac{SS}{df} \qquad [4]$$

where σ is the SD and df the degrees of freedom. One-SD limits provided a 68% confidence interval of the regression line.

RESULTS

The plasma concentration × time profile for adriamycin disposition appeared to be triphasic with mean half-lives calculated from nonlinear regression of a classic three-compartmental model (NONLIN) for the three phases of 9.2, 90.8, and 1556 minutes for all 23 patients studied. The comparisons between predicted and observed adriamycin plasma time courses for 23 individual patients following single iv bolus doses are presented in table 3. Of the 16 patients who did not have clinical evidence of either impaired liver or kidney function, 11 demonstrated reasonable agreement between their measured and model-predicted plasma time courses. Figure 2 illustrates the range of this comparison for several patients. All four patients with elevated serum bilirubin evidenced significantly higher plasma concentrations and a prolonged plasma half-life relative to the model prediction, which assumed normal liver function and drug clearance. Two such patients are depicted in figure 3. The presence of hepatic tumor in one patient (No. 1, table 3) with normal serum bilirubin did not appear to affect adriamycin clearance (fig 2a). The significance of hepatic clearance is further evidenced by the patient receiving successive 30-mg/m² doses by iv and intra-arterial bolus injection. As illustrated in figure 4, the iv course approximates the model prediction better than the intra-arterial course.

The predicted and observed plasma time curves for the two patients with impaired kidney function provided inconclusive results. In one patient, excellent agreement was observed over a relatively short time interval following administration (fig 5a). In the other patient, poor agreement was observed (fig 5b), although it should be pointed out that this patient received the highest dose administered as a single intermittent bolus, ie, 60 mg/m². The patient with impairment of both liver and renal function (No. 3, table 3) evidenced the worst fit between the predicted and observed plasma time courses of all patients studied with a mean square error of 0.422.

The plasma time course for adriamycin metabolites was found to be quite variable between patients, although adriamycinol was generally observed to be the predominant plasma metabolite. Adriamycinol plasma disappearance appeared to parallel that of adriamycin in many patients after 4 hours as seen in figure 2. In some individual patients, however, several metabolites were observed in the plasma (fig 6).

Comparison between predicted and observed adriamycin tissue concentrations from surgically removed biopsy samples from nine patients following iv bolus doses is shown in table 4. Because this

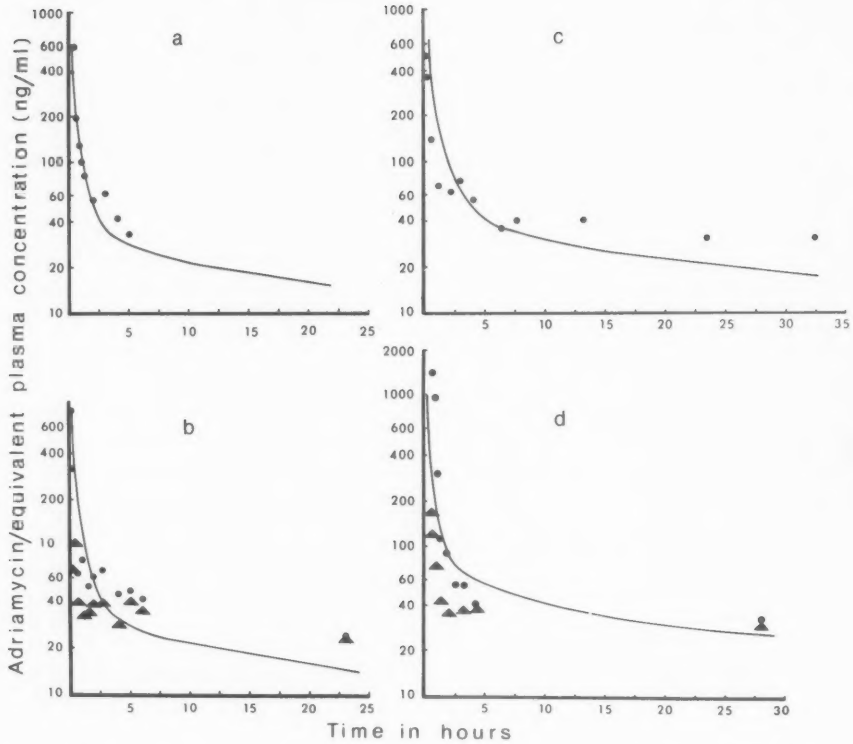

FIGURE 2.—Predicted (smooth line) and observed (●) adriamycin plasma concentrations for 4 patients [(*a*) patient 1; (*b*) patient 11; (*c*) patient 7; and (*d*) patient 8] with normal liver and kidney functions. Adriamycinol (▲) concentrations are also shown.

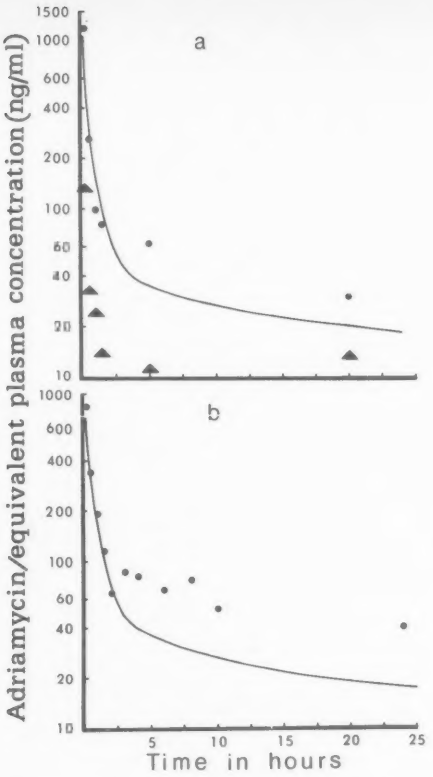

FIGURE 3.—Predicted (smooth line) and observed (●) adriamycin plasma concentrations for patients 10 (*a*) and 18 (*b*) with impaired hepatic functions. Adriamycinol (▲) concentrations are also shown.

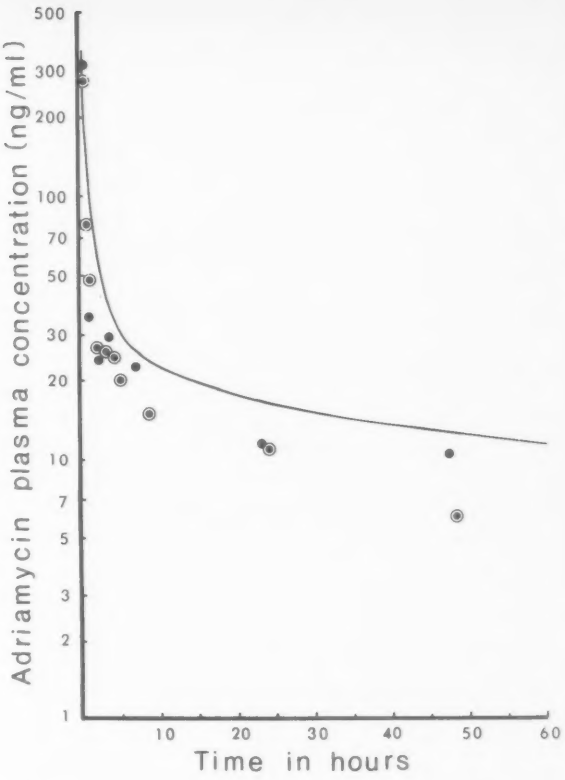

FIGURE 4.—Adriamycin plasma time course following 30-mg/m² iv (●) and intra-arterial (◉) bolus doses in patient 10. The model prediction is shown as the smooth line.

preliminary model does not have a tumor compartment, no comparisons were possible with regard to tumor uptake. Selected adriamycinol tissue concentration data are also included in table 4.

DISCUSSION

This study represents an attempt to evaluate the capability of a multicompartmental flow-limited model in predicting adriamycin disposition in several patients differing in clinical status. We chose to utilize average distribution and clearance data in the model so that an analysis of deviation between predicted and observed adriamycin concentration would allow us to identify important patient-specific characteristics about this drug that would produce a clinically useful model. A major difficulty exists, however, in assessing the relative predictability of such a model where the predicted parameters are not derived from the experimentally determined concentration × time data for either a single patient or from a group of patients resulting in an "average" patient prediction. While it is possible to quantitate the difference between the predicted and observed data for a finite number of points, as was done here in terms of a mean square error term, the quantitation of these differences cannot be evaluated by established statistical criteria. Although admittedly subjective, the qualitative comparisons made here appear to be consistent with the magnitude of differences reported for similar types of predictions for a limited number of other drugs (16,17,25).

To get a better perspective on this comparison between the predicted and observed plasma time course, the differences seen here were compared to the typical variation one could expect in fitting the data by classic compartmental pharmacokinetic techniques. If typical "noise" in the data is taken into account, a confidence interval for the computer-

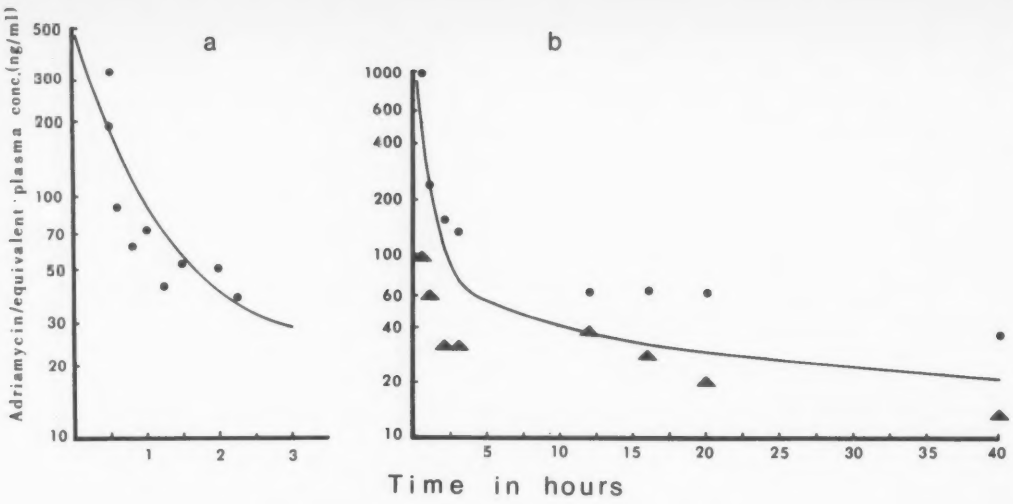

FIGURE 5.—Predicted (smooth line) and observed (●) adriamycin plasma concentrations for patients 21 (*a*) and 22 (*b*) with impaired kidney function. Adriamycinol (▲) concentrations are also shown.

calculated nonlinear regression line can be calculated. Figure 7 illustrates the 68% confidence interval for this line for a typical patient using a three-compartment model. This confidence interval represents the certainty of the classic prediction within limits of 1 SD. Calculation of a mean square error term comparing the regression line data and that deviating by 1 SD for six time points in a manner comparable to that described for the physiologic-model prediction produced values in the range 0.03–0.09 for typical patient data. This suggests that the relative fit between the actual and physiologic model-predicted data is at least of the same order of magnitude as the expected variance in the classic model data, and as such we feel justified in the somewhat subjective goodness-of-fit criteria we have defined here. In addition, accomplishing this type of prediction by modeling rather than fitting enhances our understanding of the kinetic variables for this drug. It is important to recognize that agreement between predicted and measured plasma time courses could arise fortuitously and as such does not necessarily imply accuracy of modeling. It is, however, a reasonable indirect indication of the feasibility of the approach and useful for detection of differences in individual patient variables which require inclusion into the model.

Our experimental data regarding the adriamycin plasma time course are in agreement with those previously published (8–10,21,26) demonstrating multicompartmental kinetics with a relatively long and variable terminal phase plasma half-life. The preliminary flow-limited model does appear to be reasonably consistent with the plasma kinetics of adriamycin in patients with normal kidney and liver function. Overall, the model-predicted and observed time course for adriamycin matched closely for the first 4 hours in nearly all of the 23 patients. Since this early time is considered to be in the distributive phase with respect to adriamycin disposition, this suggests that reasonable approximations concerning tissue masses, binding, and blood flows were made in construction and scaling of the model. The plateau or actual increase in plasma adriamycin concentration observed in several of the figures between 2 and 4 hours following an iv dose was seen commonly in our experimental data. Since these concentrations are well above the sensitivity limits of the assay, this is apparently a real, rather than an anomalous, effect. Close examination of the data of Benjamin et al (8,26) indicates similar patterns in the plasma time curve. Neither the classic three-compartmental nor the physiologic model accounts for this pattern and previous data indicating poor oral absorption of adriamycin (12) would taint any explanation involving biliary excretion and enterohepatic recycling.

The increased deviations at later times in some "normal" and most liver/renal impairment patients further suggest that the model does not fully account for individual differences in metabolism and/or clearance and that patient-specific improvements

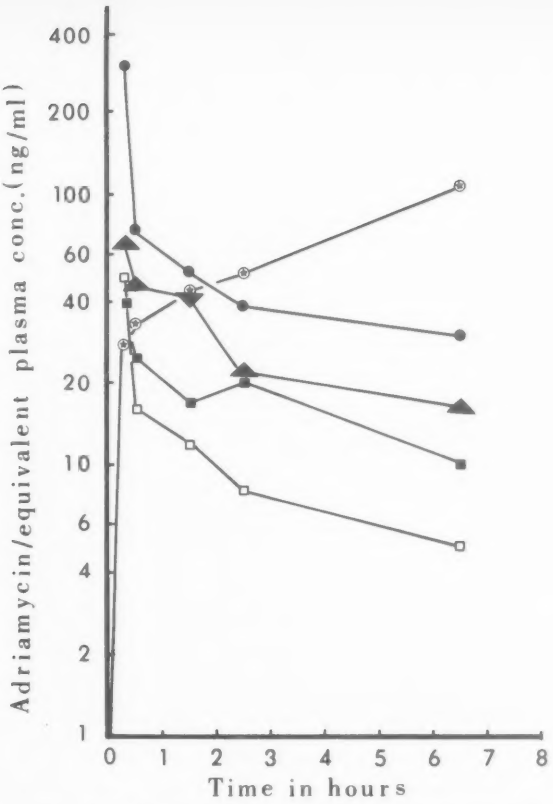

FIGURE 6.—Adriamycin (●) and metabolite concentrations in patient 13 following a 40-mg/m² bolus iv dose. ▲ = adriamycinol; ⊙ = unidentified polar metabolite; and □ and ■ = unidentified aglycones.

must be incorporated in these terms. Data from our own laboratory suggest significant individual differences in metabolic rate and profile (27) and other investigators have reported widely varying terminal log-linear half-lives (9,10,19). In addition, analysis of the data of Creasey et al (19) indicates a possible increased half-life for patients receiving 90-mg/m² iv bolus doses relative to that for lower doses. We have observed similar variability in a small number of patients receiving 60-mg/m² doses; however, the differences were not statistically significant. Of the five patients with normal liver and kidney function for whom there was poor agreement between observed and predicted plasma time courses, four evidenced adriamycinol plasma concentrations greater than adriamycin following the distribution phase and the actual adriamycin clearance observed was more rapid than that predicted by the model in all four of these patients. This apparently variable metabolic clearance suggests that rate constants for

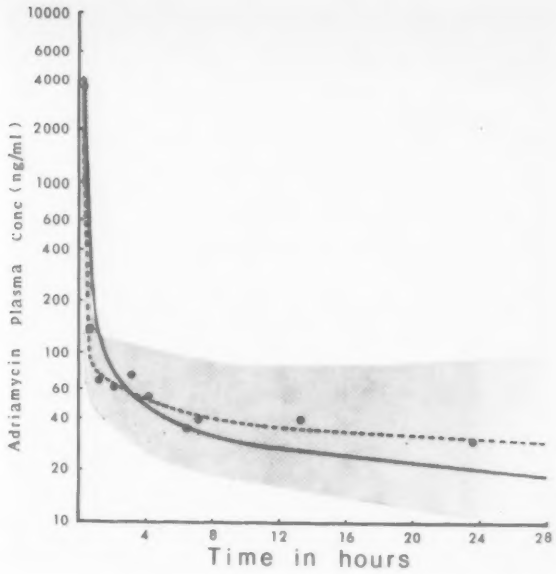

FIGURE 7.—NONLIN (*from* ref 22) fit (broken line) of adriamycin plasma concentration data to a 3-compartment open model in patient 7. Shaded area indicates ± 1 SD of the fit. The model-predicted plasma time course is also shown (solid line).

adriamycin metabolism and biliary excretion may be necessary to include in the model in place of the present total hepatic clearance term.

Consistent with previous reports (5), all four patients with significant hepatic dysfunction as measured by elevated serum bilirubin evidenced elevated, prolonged adriamycin plasma levels relative to those predicted by the model. More patients are required to quantitate the relationship between clearance and bilirubin as well as an understanding of the effect of liver disease on the biliary excretion and metabolic pathways. In order for this type of model to allow a prediction of adriamycin disposition, these observed variations in clearance, even in patients with normal hepatic function, must be accounted for. Additionally, the possible clinical and/or toxic effects of adriamycin metabolites, specifically adriamycinol (26), must be examined in light of the prolonged half-life and significant plasma levels observed.

Our data do not allow any conclusions regarding impaired renal function to be drawn. Only two patients were studied, one for a very short time and the other at high dose levels. In addition to its possible drug clearance function, the kidney is a very highly perfused tissue and substantial adriamycin uptake has been demonstrated in rabbits (12). Based upon this and the recent report by Creasey et al (19), the kidney may be responsible for a

TABLE 4.—Adriamycin tissue concentration data for 9 patients following an iv bolus dose

Patient No.	Dose (mg/m²)	Disease	Time (hrs)	Tissue adriamycin	Model* (μg/g)		Tissue adriamycinol (μg/g)
T-1	30	Melanoma	4	Lymph nodes	0.88 †		—
T-2	30	Colon carcinoma	4	Tumor	9.2 †		—
T-3	30	Soft tissue sarcoma	3	Tumor	2.14 †		—
				Shoulder (muscle)	1.63	0.27	—
T-4	30	Breast carcinoma	2	Tumor	2.49 †		—
				Node	2.60 †		—
				Muscle	0.37	0.45	—
				Skin	0.29	0.45	—
T-5	30	Colon carcinoma	1.5	Lymph nodes	3.06 †		—
				Colon	1.54	3.33	—
			2.5	Breast (muscle)	1.58	0.25	—
				Colon	1.07	2.92	—
T-6	30	Liver carcinoma	4	Tumor	0.60 †		0.22
				Tumor	0.36 †		0.22
				Tumor	0.65 †		0.23
				Normal liver	2.29	1.42	0.38
T-7	30	Soft tissue sarcoma	2.5	Fat	0.15	0.37	0.13
				Fat	0.21		0.13
				Skin	0.52	0.32	0.08
				Skin	0.28		0.19
				Muscle	0.36	0.32	0.06
				Muscle	0.33		0.12
				Marrow	0.52	1.37	0.12
				Tumor	0.50 †		0.37
				Tumor	0.39 †		0.10
T-8	30	Hepatoma	2	Tumor	3.51 †		0.58
				Normal liver	2.47	1.7	1.3
T-9	30	Soft tissue sarcoma	3.5	Fat	0.33	0.28	0.34
				Fat	0.33		0.33
				Muscle	0.41	0.27	0.26
				Muscle	0.44		0.19

* Predicted value from multicompartmental model.
† Not included in model.

greater fraction of adriamycin clearance than previously thought, and more patients are being studied.

Perhaps the major utility of a physiologic model is the potential to predict drug levels in tissues which can not be readily sampled. A good predictive fit of tissue data would also lend support to the appropriateness of the model design and choice of parameters. Although extensive tissue data are difficult to obtain from an individual patient, our data for selected samples from nine surgery patients indicate reasonable agreement. All the predictions were within an order of magnitude, and poorly perfused tissues such as fat and lean tissue showed very good correlations. The greater degree of variability observed in tissues with higher relative blood flow may be due to variation in tissue condition, blood flow, and tissue binding in and around the tumor—which is where all samples were obtained. The overall reasonable fit for these samples, all removed during the first 4 hours following drug administration, further supports the initial estimates of blood flow, tissue mass, and drug-binding parameters utilized in model construction. Although it would be highly desirable to include a tumor compartment in the model, the uncertainties of size, blood flow, and binding capacity and the large number of different tumors against which adriamycin shows activity precluded this in the preliminary model. It will, however, be incorporated in a refined model and should enhance the approach.

While it is unlikely that a ten-compartment model will be necessary to account for patient-specific variables, it seems reasonable to include all areas of the body which demonstrate different tissue/plasma equilibrium ratios of adriamycin uptake in construction and assessment of fit. For subsequent clinical use, it can be simplified by combining compartments which appear not to be patient-specific to avoid unnecessary complexity in interpreting the data or providing input for computer analysis.

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Phase I Study of Hycanthone [1,2]

Sewa S. Legha,* William E. Grose, and Gerald P. Bodey[3]

SUMMARY

Hycanthone was given to 15 patients with metastatic cancer in order to determine the maximum tolerable dose. The drug was administered in 5-day courses at 3-week intervals. The starting dose was 30 mg/m²/day and the highest dose level reached was 90 mg/m²/day. The most common (13 patients) side effect was nausea and/or vomiting. The dose-limiting toxicity was toxic hepatitis manifested as elevation in serum transaminases in eight of 15 patients and an increase in serum bilirubin in three patients. Hepatotoxicity was dose-related and was observed in two of 25 courses given at the dose level of ≤ 70 mg/m² compared to seven of nine courses given at the dose level of ≥ 80 mg/m². Because of an unacceptable incidence of hepatotoxicity at higher doses, 70 mg/m²/day × 5 appears to be a safe dose for phase II studies.

[Cancer Treat Rep 62:1173–1176, 1978]

Hycanthone is a compound with potent antischistosomal activity that has been used in the treatment of infections with *Schistosoma mansoni* and *Schistosoma haematobium* since 1968 (1). In structure, hycanthone is a hydroxymethyl derivative of lucanthone (miracil D), an antischistosomal agent which has been in use for several decades. David Rosi (Sterling Winthrop Research Institute) first prepared the drug by exposing lucanthone to the enzymatic activity of *Aspergillus sclerotiorum* (2). Subsequently, hycanthone was found to be the biologically active metabolite of lucanthone detectable in the urine of several animal species and patients following treatment with lucanthone.

Because of its structural similarities to clinically useful antitumor drugs such as actinomycin D and daunorubicin, hycanthone was investigated as a potential antitumor agent against the animal tumors (3). Hycanthone was found to be cytotoxic to several tumor cell lines in tissue culture and exerted significant antitumor activity in vivo against Walker 256 carcinosarcoma, L1210 leukemia, and P815 mast cell tumor (4). Mechanism studies have shown that hycanthone inhibits both DNA and RNA, but does not inhibit protein synthesis in human phytohemagglutinin-stimulated lymphocytes (4). It has been proposed that hycanthone binds to DNA by intercalation (5).

There is extensive experience with the use of hycanthone in the treatment of schistosomiasis. In the commonly used 3-mg/kg single im dose schedule, it is curative in 80%–90% of the patients. The side effects observed with this dose schedule include frequent nausea and vomiting and, less commonly, abdominal pain, headache, and dizziness. Rare cases of hepatotoxicity including death from hepatic necrosis have been described (1,6,7). Animal toxicology studies carried out at the National Cancer Institute have revealed gastrointestinal and hepatic toxicity to be the major toxicity from hycanthone. Hepatic toxicity manifested mainly in the form of an increase in serum transaminase levels and it was not cumulative on repeated exposure. This compound also produced neurotoxicity in the form of ataxia, muscular weakness, and convulsions; however, even at lethal doses, it had no myelosuppressive toxicity. The monkey was more sensitive than the dog and

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the dose producing the minimal degree of toxicity (toxic dose low) was 100 mg/m²/day × 5 days. Based on the experimental evidence of antitumor activity and predictable toxicity, a phase I study was carried out in our department. Since the experimental antitumor activity was not schedule-dependent and the toxicity data in the monkey were taken as the basis for choosing the starting dose in man, a daily × 5 schedule of drug administration was selected for this study.

MATERIALS AND METHODS

Adult patients with advanced neoplastic diseases refractory to prior therapy who were not eligible for higher priority protocols were entered in this study. Eligible patients had recovered from the toxicity of previous therapy and had normal blood counts and hepatic and renal functions. However, selected patients with a minor abnormality of hepatic or renal function, presumably from neoplastic involvement, were also eligible. Patients with a serum bilirubin level above the normal range were specifically excluded. Informed consent was obtained from all patients according to institutional policy.

The starting dose of hycanthone was 30 mg/m²/day × 5 days. Subsequently, the dose was escalated by 50% if no toxicity was observed. After determining the dose causing minimal toxicity, the dosage during subsequent courses was escalated by 25%. Courses of therapy were repeated at 3-week intervals. The drug was dissolved in 100 ml of 5% dextrose solution and was infused iv over a period of 45–60 minutes.

Patients were monitored for toxicity and potential antitumor activity. A cbc count including hemoglobin and wbc and platelet counts was obtained once weekly and prior to each treatment. Liver and renal functions were checked twice weekly. Changes in measurable disease were recorded prior to each course of therapy. Repeated courses of treatment were given provided there was no progression of tumor between courses. Dose escalations were carried out in individual patients provided there was no toxicity from the previous course. Patients were taken off the study if there was progression of tumor after a minimum of two courses of treatment. Hycanthone therapy was discontinued any time a patient developed liver dysfunction accompanied by an increase in total serum bilirubin level > 1.5 mg/100 ml (normal range, 0.15–1.0 mg/100 ml).

RESULTS

A total of 15 patients received 35 courses of hycanthone treatment between April 1977 and December 1977. A summary of various dose levels used and the number of courses given at each dose level including the side effects is shown in table 1. The distribution of patients by various tumor types was as follows: adenocarcinoma, lung (two patients); epidermoid carcinoma, lung (two patients); adenocarcinoma, colon (three patients); adenoid cystic carcinoma, parotid gland (two patients); adenocarcinoma, unknown primary (one patient); and melanoma, neuroblastoma, mesothelioma, and adenocarcinoma, kidney and urethra (one patient each). There were three women and 12 men with a median age of 54 years (range, 20–72). All except the single patient with adenocarcinoma of unknown primary site were previously treated with chemotherapy.

Gastrointestinal toxicity was the most common side effect and was observed in 13 of 15 patients (87%). It manifested as anorexia, abdominal pain, nausea, and, less frequently, vomiting (five of 15 patients, 33%). The vomiting was generally limited to one or two episodes and had stopped by the last day of the 5-day course of treatment. Normal appetite generally returned by 1 or 2 days after the course of treatment was completed.

The dose-limiting toxicity was hepatic and was observed in eight of 15 patients (53%). It generally consisted of elevation of transaminases and mild elevation of alkaline phosphatase. The increase in transaminase levels was variable, ranging from 20 to 2000 IU (normal, 5–20 IU). Hepatic toxicity was clearly dose-related with only two episodes among 25 courses (8%) given at ≤ 70 mg/m² compared to seven episodes among nine courses (78%) given at ≥ 80 mg/m². The earliest abnormality in liver enzymes was observed on Day 3 or 4 of a 5-day course; the enzyme level reached maximum on Day 7 and returned to normal by Days 10–14. Three patients developed an increase in serum bilirubin (at 80, 90, and 90 mg/m² respectively). One of these patients reached a maximum bilirubin elevation of 1.6 mg/100 ml on Day 10 of the course, which returned to normal by Day 15. Two patients developed jaundice with peak bilirubin levels of 20 mg/100 ml each. One of these patients gradually improved with the bilirubin level dropping down to 3 mg/100 ml over a period of 60 days. The other patient (at an 80-mg/m² level) gradually deteriorated and died of hepatic failure on Day 15 of his first course of chemotherapy.

Hycanthone therapy was discontinued in four patients after a single course of therapy, three because of disease progression and one because of hepatotoxicity. Eleven patients received repeated courses of therapy with a median of three cycles per patient (range, two to five cycles). Four of the six patients treated at the dose range of 30–45 mg/m²/

TABLE 1.—Dose escalation scheme and toxicity of hycanthone

Patient No.	Dose level (mg/m^2/day × 5 days) Course No.— 1	2	3	4	5	Toxic effect
1	30					Profuse vomiting
2	30					Nausea
3	30	30				Anorexia, headache
4	30	45				None
5	30	45				Anorexia, nausea, abdominal pain
6	30	45	70	90		Anorexia, nausea, jaundice after course 4
7	70	70	70	70	70	Nausea, increase in SGOT with course 3 (normal thereafter)
8	70	70	70			Anorexia, nausea, vomiting
9	70	70	70			Nausea, increase in SGOT during course 3 only
10	70	70	80			Nausea, vomiting
11	70	90				None
12	80					Anorexia, nausea, jaundice (drug-related death)
13	80					Vomiting, increase in SGOT
14	80	80*				Vomiting, increase in SGOT (both courses)
15	90	80*	80*			Vomiting, bilirubin 1.4 mg/100 ml with course 1, increase in SGOT with courses 2 and 3

* mg/m^2/day × 3 days.

day received repeat courses (three at a dose of 45 mg/m^2 and one at a dose of 70 mg/m^2/day). None of these patients developed liver function abnormalities. Three of the five patients who began initial courses at 70 mg/m^2/day received repeat courses at 70 mg/m^2 (three, three, and five courses respectively) and two subsequently received escalated doses of 80 and 90 mg/m^2 respectively. Two patients developed mild elevation of SGOT (to twice the normal level), both during the third cycle at 70 mg/m^2. Neither of the patients who were subsequently escalated to 80 and 90 mg/m^2 had liver toxicity.

Three patients received hycanthone at the 90-mg/m^2 dose level. Two of these patients had initially received treatment at lower dose levels; the third began his initial course at 90 mg/m^2. One patient had no liver toxicity at this dose level (one course). The second patient developed jaundice and received no further chemotherapy. The third patient, who received the initial course at 90 mg/m^2, developed mild hepatitis (bilirubin increased to 1.4 mg/100 ml). Her liver function normalized within 2 weeks and she subsequently received two more courses at 80 mg/m^2/day × 3 days with only a minor increase in serum transaminases.

In a few patients, other toxicities observed concomitant with hycanthone therapy included such nonspecific symptoms as headache, dizziness, and generalized weakness. Two patients, both at the dose level of 90 mg/m^2, were observed to have a decrease in systolic blood pressure of 40–50 mm which lasted about 15–20 minutes before returning to normal. The rate of infusion in these patients was the same as that in the remainder of the patients and no other cause for hypotension was obvious. There was no myelosuppression at any dose level. There was no evidence of phlebitis or tissue injury on extravasation of the drug.

No obvious tumor regression was observed. Two patients with adenocarcinoma of the colon may have had some antitumor effect. One of these patients had rapidly advancing pulmonary metas-

tases that stabilized for a period of 3 months. This was accompanied by a 30% decrease in the carcinoembryonic antigen level (from 3400 to 2100 ng). The other patient with a pelvic mass showed some shrinkage of the tumor accompanied by significant relief in the abdominal pain. A single case of pleural mesothelioma had significant subjective improvement accompanied by some decrease in the pleural thickening, but the patient was taken off the therapy because of hepatic toxicity. Another patient, with metastatic adenocarcinoma of the parotid gland, had stabilization of her disease with no change in the size of tumor metastases for 2 months.

DISCUSSION

From this phase I study, the maximum tolerated dose of hycanthone appears to be 70 mg/m^2/day × 5 days at 21-day intervals. Minimal side effects were observed at this dose level and consisted of anorexia, nausea, and occasional vomiting. Only two of 15 courses given at this dose level caused a slight increase in the liver enzymes. The incidence and severity of toxicity, however, increased steeply at the higher doses and toxic hepatitis was the dose-limiting toxicity. Hepatic toxicity was acute, and became obvious within 48–72 hours with a sharp rise in transaminases. The enzyme elevations rapidly normalized unless the liver damage was considerable in which case hyperbilirubinemia developed. There was considerable individual variation in tolerance to hycanthone. Since all of our patients had normal liver functions prior to hycanthone administration, liver function abnormalities did not account for this variability. With the limited number of courses given per patient (because of short survival or because of progression of tumor in the interim), it is not possible to make a clear statement regarding cumulative hepatic toxicity of this drug.

The most remarkable feature of hycanthone was its total lack of myelosuppression. This would make it a suitable drug for combination chemotherapy if it is found to have even a low order of antitumor activity in further clinical testing. Hepatic toxicity of hycanthone is its major drawback, but since it is infrequent at the dosage recommended for phase II studies, hycanthone should at least be screened against some of the common tumor types. Daily determination of liver enzymes is necessary during hycanthone administration and if the transaminases show a significant rise during the course of treatment, therapy should be immediately discontinued. Since there is some evidence that hycanthone is metabolized by liver and excreted through bile (8), caution is necessary in patients with liver disease or hepatic metastases. The use of other hepatotoxic agents such as phenothiazine antiemetics should be avoided in patients receiving hycanthone.

Although we have used hycanthone in a 5-day schedule, it is possible that a different schedule of administration may be less hepatotoxic. Pharmacologic studies may be of help in this regard and we hope to obtain some leads from the limited data that we have gathered during our phase I study.

Another area of potentially fruitful investigation with hycanthone is its possible application as a radiosensitizer. This is based on the radiosensitizing properties of the parent compound lucanthone (9).

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An Immunologic Look at the TNM Classification: Therapeutic Implications and Strategies [1]

Lucien Israel [2,*]

Ever since the TNM classification was devised, histologically proven invasion of lymph nodes by cells from a primary tumor has been considered as an extension of that tumor both in space and time. In other words, lymph node involvement has generally been viewed as a phenomenon reflecting the fact that tumor cells have travelled the *distance* between the primary tumor and the regional lymph nodes and that a given *interval* was required for this to occur. It is a well-established fact that this invasion of lymph nodes by tumor cells bears definite prognostic significance. Consequently, anatomic extension soon assumed major prognostic importance and the TNM classification came to be considered as a *static* indication of this extension and a tool for evaluating postsurgical prognosis when no postoperative treatment was undertaken.

Aside from the growth rate of a tumor, a dynamic factor not taken into account by the TNM classification, I would like to put forward the idea that lymph node invasion by tumor cells is an immunologic phenomenon rather than an anatomic one, and that this implies the use of novel therapeutic approaches. This suggestion is based on experimental and clinical data from the literature as well as on a number of hypotheses which will be presented as such. I am well aware that this view, by its very nature, is bound to be controversial, but I would nevertheless like to submit it as it stands for consideration by the community of medical and surgical oncologists.

EXPERIMENTAL DATA ON REGIONAL LYMPH NODES

Fisher et al showed that the purpose of regional lymph nodes is not to act as a filter (1,2), but rather to fulfill an immunologic function (3–5). These authors demonstrated that, in the presence of a tumor, lymphocytes from regional lymph nodes exhibited a stronger response to phytohemagglutinin than that observed when tumor was not present. It was also shown that in tumor-bearing animals, lymphocytes from regional lymph nodes responded better to this mitogen than did peripheral lymphocytes or lymphocytes from distant lymph nodes.

It has recently been shown that the immunologic reaction that takes place in the regional lymph node is not a purely nonspecific one. The work of Goldfarb and Hardy (6) revealed that the specific cytotoxic response of lymphocytes to autologous tumor cells was even more markedly enhanced than the nonspecific response. The same authors also demonstrated that the specific cytotoxic response of regional lymph nodes could be detected as early as the third day following a tumor graft, reaching a peak between Day 7 and 21 and then gradually declining. The same phenomenon was seen, but was delayed in splenic lymphocytes as well as in lymphocytes from distant lymph nodes, though the cytotoxic response was weaker in the latter case. Investigations into the consequences of regional lymphadenectomy on tumor growth have confirmed the importance of regional lymph nodes. Pendergrast et al (7) and Pilch et al (8), while confirming the interesting results of Alexander et al (9), showed that regional lymphadenectomy influenced neither the growth of the primary tumor nor the natural history of metastatic spread once a tumor had become clinically detectable, whereas it had a detrimental effect when performed prior to or < 2 weeks after a tumor graft. All these data suggest that it is in the lymph nodes draining a primary tumor site that host "immune instruction" with regard to specific tumor antigens occurs. This "knowledge" is then transferred systemically and can later be detected at more distant sites. When "immune instruction" does not take place, there is little if any transmission of information and the host is less apt to control systemic disease.

The studies quoted above are not very explicit with regard to the role of macrophages either as vectors of nonspecific immunity or as agents of

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antigen processing. Similarly, the role of natural killer cells remains to be elucidated. Despite these shortcomings, the fact remains that today it has become impossible not to attribute a role of the utmost importance to regional lymph nodes in the development of antitumor immunity, both specific and nonspecific. These data can be summarized by saying that nonspecific and specific antitumor immunity arises in the regional lymph nodes very early in the process of tumor development and tends to progressively disappear as the tumor grows. The appearance and development of tumor foci within these lymph nodes must be considered in light of these phenomena. A study by Flannery et al (10) has shown, furthermore, that tumor growth induces a state of gradually increasing unresponsiveness in regional lymph nodes.

CLINICAL DATA ON REGIONAL LYMPH NODES

It has been known for a long time that regional lymph node status at the time of removal of a primary tumor is of prime prognostic importance. This has proved to be true for the vast majority of epithelial tumors and has served as a basis for classifying patients into numerous subgroups.

More recently, pathologists and clinicians have drawn attention to the prognostic significance of lymphocyte function in regional lymph nodes. Ellis et al (11) showed that in patients with operable breast cancer, regional lymph node lymphocytes exhibited a stronger response than peripheral lymphocytes in the migration inhibition assay using autologous tumor antigen. Fisher et al found a similar difference using nonspecific stimulation with phytohemagglutinin (12). In contrast, Humphrey et al (13) did not find this phenomenon using the response to tetanus toxoid.

There is a growing consensus as to the significance of the histopathologic picture in the regional lymph nodes of cancer patients. Following the work of Black et al (14,15), who drew attention to the good prognosis associated with sinus histiocytosis in the regional lymph nodes of breast cancer patients, Berg et al (16) showed the importance of reactive hyperplasia in these nodes. These findings have recently been confirmed by Fegiz et al (17) and similar observations have been reported by Van Nagell et al (18) for carcinoma of the uterine cervix in which it was shown that prognosis improved from the lymphocyte-depleted nodal pattern to the lymphocyte-predominant nodal pattern, whereas the unstimulated or germinal-center-predominant pattern was associated with a prognosis intermediate between the two. Pihl et al (19) showed that in colorectal carcinoma, the degree of sinus histiocytosis of resected regional lymph nodes correlated well with the in vitro cytotoxicity of lymphocytes from these lymph nodes against autologous tumor cells. This correlation with cytotoxicity was also found in cases of either T cell hyperplasia or B cell hyperplasia, but was even more striking when combined B and T cell hyperplasia was present. These findings assume even greater significance if considered in light of an earlier study by Pihl et al (20) showing that these histopathologic features were associated with a favorable prognosis. Similar findings have been reported by Patt et al (21) and many other investigators.

At this point, I would like to add a personal finding which is relevant to the above data. In 1973 we showed (22) that among 323 patients with adequately resected bronchogenic squamous cell carcinoma, the median survival of 165 patients whose preoperative purified protein derivative (PPD) skin test was positive was > 16 months while the median survival of 158 patients with a negative preoperative PPD skin test was only 8.6 months. In the latter group, 33 patients spontaneously converted their response to positive postoperatively; the median survival of these 33 patients was 14 months. In a recent survey it was noted retrospectively that 174 patients had stage I disease, as defined in the recent study by McKneally et al (23), ie, no lymph node involvement or simply microscopic involvement confined to the hilar nodes, virtually contiguous with the primary tumor. Thirty-four of these 174 patients (20%) were among the PPD-negative patients and were destined to have a short survival whereas 140 (80%) were in the PPD-positive group and had a longer survival.

From the studies quoted it is not perfectly clear what the possible role of limited, microscopic lymph node metastases might be. It seems clear that while the absence of lymph node involvement implies a better overall prognosis, very limited involvement does not necessarily preclude a strong specific cytotoxic response. This is not the case when massive involvement is observed. At this point, it must be mentioned that in all surgical series, 10%–20% of patients with no lymph node metastases nevertheless have a poor prognosis and their tumors are destined to metastasize or spread rapidly despite this absence of initial lymph node involvement.

In general, however, the above findings demonstrate that in human cancer there is a close, though not absolute correlation between prognosis, nonspecific host immune status (the important observations of Eilber and Morton [24] must be mentioned here), specific lymphotoxic effects in the regional lymph nodes at the time of removal of the primary

tumor, and the presence of histologic features in these lymph nodes reflecting an immunologic reaction.

REFLECTIONS AND HYPOTHESES

In light of all the experimental and clinical data discussed above, I would like to present a number of reflections and tentative answers to the questions which are raised.

What does N^+ Mean?

It appears quite clear that the N^+ status does not reflect anatomic extension but essentially it reflects immune deficiency. Viewed from this angle only, the TNM classification is incongruous in that it combines two "static," anatomic features with one "dynamic," immunologic characteristic. (Why not, then, also include the growth rate, a very important prognostic factor?) If the N^+ status does indeed reflect an immune deficiency, what then is the relative contribution of the specific and nonspecific components to the deficiency? This is difficult to answer with certainty. It may be noted, however, that if nonspecific processes such as inflammation, macrophage activation, antigen-processing, and lymphokine secretion are inhibited, there is little chance of the specific immune response taking place and being expressed in vivo.

What does N_0 mean?

As mentioned earlier, the absence of lymph node metastases cannot reasonably be conceived to signify that tumor cells have not had time to travel the distance between the primary tumor and the regional lymph nodes. In this regard, it should be recalled that specific lymphotoxicity, which implies contact with the antigen, can be clearly detected as early as 3 days after an experimental tumor graft. Two explanations may be suggested to account for the N_0 status: either the tumor cells have crossed the lymph node without being stopped (this group of what I shall call "bad N_0s," which represents approximately 20% of cases irrespective of site, may correspond to the "unstimulated pattern" described by Pilh, which will be discussed later), or tumor cells reaching the regional lymph nodes are destroyed there by specific and possibly nonspecific mechanisms (nonspecifically activated macrophages). Thus, in most cases, the N_0 status may be viewed as an indication of adequate host-immune response and considered to represent a potential mechanism for biologic control of tumor spread. On the other hand, although every oncologist has seen large primaries without lymph node involvement, it is true that N status and tumor size correlate reasonably for many cancer sites, or, in other words, that there is some correlation between N status and growth rates. In the light of the theory proposed in this paper, this correlation could be explained by the weakness of the immune attack against the primary.

What are the causes of "bad N_0s"?

From a theoretic standpoint, it is obviously frustrating to encounter bad N_0s. I would like to mention here a clinical "impression" shared by several of my colleagues that the clinical course of bad N_0s is particularly hopeless and rapid. I should apologize for the imprecision of the term "impression," though, to the best of my knowledge, this has not been documented by any statistical series. Two hypotheses, which are not mutually exclusive, may be put forward to account for this phenomenon. The first is that tumor antigens may be weakly expressed or "masked" and the second is a preexisting immune deficiency state, either congenital or acquired. In both cases, "immune instruction" fails to take place in the regional lymph node which lets tumor cells through without responding, neither biologically nor histologically.

Why do N_0s eventually become N^+s?

This is one of the most important questions that must be answered if the theories developed in this paper are to be given any weight. It is true that the incidence of N^+ rises as the interval between clinical detection and surgical removal of a primary tumor becomes longer. It is also true, as noted by Flannery et al (10), that the immune response in regional lymph nodes tends to grow weaker as time goes by, as does the systemic immune response. Why does the immune response fade once it has developed? In a number of personal studies (22,25,26) we have highlighted the fact that immune deficiency in cancer patients could be ascribed to a paraneoplastic syndrome, that is, a biologic syndrome directly or indirectly controlled by the tumor itself which uses these disturbances as a means of protecting itself against host-immune mechanisms. We have recently drawn attention to the high levels of acute phase proteins found in cancer patients and their correlation with disease stage. These acute phase proteins, synthesized by the liver in response to a signal from the tumor, exert nonspecific immunosuppressive properties in vitro (27,28). Such "nonspecific blocking factors of host origin," as we have called them, are essentially sialoglycoproteins rich in sialic acid, capable of masking binding sites on lymphocytes and macrophages. It is our opinion that the high circulating levels of these factors in

some cancer patients account for the immune inertia observed in a number of cases. Furthermore, reduction of serum levels of these factors by plasmapheresis (28–30) has enabled us to observe transient partial tumor regressions, conversion of several previously negative delayed hypersensitivity skin tests to positive, and a drop in the third component of complement, presumably due to overconsumption.

In addition to the nonspecific factors described in the above studies, a number of "nonspecific blocking factors of tumor origin" have been described although the concept itself has not been clearly expressed (31–33). These various factors, essentially polypeptides, also induce immune paralysis or immune "indifference." Concentrations of these factors are likely to be higher in regional lymphatic vessels and lymph nodes than in the systemic circulation. It may be postulated that these agents represent one of the important factors underlying progressive nonspecific immunologic failure in regional lymph nodes. This nonspecific deficiency then presumably prevents the expression of the specific response.

Last but not least, the role of immune complexes should not be underestimated (34,35) as they probably represent the true "specific blocking factors" and it is conceivable that their concentration might also be higher in regional lymph nodes where tumor cells and immunocytes are continually coming into contact with each other (as well as in the tumor's immediate environment from where these complexes are partially drained by the lymphatic circulation). Consequently, we have hypothesized that a declining regional immune response is due to a combination of three factors: nonspecific blocking factors of host origin, sialoglycoproteins that mask reactive sites; nonspecific blocking factors of tumor origin, essentially peptides that regulate or inhibit chemotaxis; and specific blocking factors, namely, immune complexes, formed either in the presence of excess antigen or excess antibody (masking either immunocyte receptors or tumor membrane antigen determinants). In such a situation, lymph node invasion by tumor cells implies that the host is no longer capable of halting either regional tumor growth or more distant extension, and this does indeed appear to be the case in the light of all the statistical series available.

THERAPEUTIC IMPLICATIONS

Although it is not the purpose of this paper, I do not wish to elude the problem of routine resection of noninvolved lymph nodes for staging purposes. This is a highly controversial question. In their experimental system, Pilch et al (8) showed that these nodes served no purpose once the tumor was established, a very important point. Crile (36) has long been in favor of conserving these nodes. In my opinion, it seems extremely important to reach, in each case, a precise staging, since, as it will be seen below, an individualized strategy is proposed for the three different categories (bad N_0, good N_0, and N^+). On the other hand, it is well known that some N_0 cases may show local recurrences from microscopic foci that were surrounding the primary at the time of its resection. Would these local recurrences have been followed in the same way by distant metastases if the regional lymph nodes had been removed or not? This is a question which could probably be answered within a short time. If such a study were to show that regional lymph nodes have a positive role to play, even in this quantitatively limited situation, it would be the task of surgeons and pathologists to find a practical answer to this problem.

How should "bad N_0s" be managed?

The first step would naturally be to identify these cases, by singling out the unstimulated lymph node patterns, studying the host's overall immunologic status, and possibly determining the antigenicity of his tumor. Indeed, bad N_0s should, in my opinion, be considered as M^+ and treated accordingly, ie, using the most aggressive forms of therapy possible.

How should "good N_0s" be managed?

It is not yet known if these cases derive any benefit from chemotherapy since ongoing adjuvant trials have all been confined to N^+ cases. Presently, I would favor postponing chemotherapeutic trials, unless "nonimmunosuppressive" agents are used, and promoting trials using specific immune stimulation at distant sites from the primary tumor combined with regional and systemic nonspecific immunostimulation. It is noteworthy in this regard that the studies of McKneally et al (23), as well as our personal studies (37), have shown that it is mostly, not to say exclusively, in patients with adequate immune function that nonspecific immune stimulation has proved its usefulness. Patients recovering to a good nonspecific immune status after tumor resection should be included in this group.

How should N^+ patients be managed?

From the above discussion, it follows that patients in the N^+ situation must be considered to be fighting a losing battle. These patients should logically receive chemotherapy, which has been shown, by

the work of Fisher et al (38) and Bonadonna et al (39), to achieve better results than followup without treatment, although the best results have been achieved when only a small number of lymph nodes are involved.

As mentioned earlier, immunodepressed patients derive little benefit from immune stimulation. If our hypothesis concerning the causes of immune depression in cancer patients is correct, then one may speculate about modalities of immune manipulation in patients with a resected N^+ tumor. The first logical step would be to attempt to remove specific and nonspecific blocking factors, whatever their origin, by repeated plasmapheresis or any other method, followed by systemic nonspecific immune stimulation and specific immune stimulation, for example, using neuraminidase-treated tumor cells as suggested by Bekesi et al (40). Only then would chemotherapy be administered using a yet to be determined schedule of interspersion with immune stimulation.

CONCLUSION

Obviously, these proposals must be submitted to controlled trials, but it is my opinion that the time has come to adopt new attitudes and new concepts to guide our therapeutic strategies. Among these concepts, that of immune manipulation, although still highly controversial, should occupy an important position. All the data collected over the past 30 years concerning the TNM classification do in fact point in this direction. These new concepts can be summarized as follows:

(*a*) The N_0 situation, in a patient with a good immune status (as assessed by skin tests) and a favorable histologic pattern in the lymph nodes, is potentially propitious for biologic control of the tumor by the host. The logical therapeutic strategy to put to trial in such a situation is stimulation of host defense mechanisms, both in terms of specific and nonspecific immunity.

(*b*) The N_0 situation, in a patient with a poor general immune status and/or with an unfavorable histologic lymph node pattern, must be considered as serious as an M^+ situation and treated as such.

(*c*) The N^+ situation means that, due to progressive weakening of the local immune response, tumor cells are able to invade the very structures whose role was to mount a local and general immune reaction against the tumor antigens. Such a situation requires systemic chemotherapy and also warrants attempts to restore the immune response jeopardized by tumor growth and subsequent stimulation of the host's immune mechanisms.

(*d*) The nodal status not only reflects the immune status of the host but also clearly reflects the extent to which a particular tumor can protect itself against the immune attack. It is likely that provided the immune status is the same, a tumor equipped with good escape mechanisms (antigen masking glycoproteins, complement inhibitors, chemotactic inhibitors, etc) will overcome more rapidly the host defenses and invade more rapidly the regional lymph nodes than a less "gifted" tumor.

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Liver Scanning in Patients With Malignancy: Rationale, Results, and Limitations [1]

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The liver is a frequent site of metastasis for many types of malignancy, and the early diagnosis of hepatic metastasis is extremely difficult. By the time the patient develops symptoms or hepatomegaly, the metastases are usually very large. More than 2 decades ago, when hepatic scintigraphy was not available, suspicion of liver metastasis was usually based on history, physical examination, and liver function tests. Although angiography and portovenography can be utilized, hepatic scintigraphy is readily available and probably more accurate for the early detection of hepatic metastasis. With the availability of newer diagnostic methods such as ultrasonography and computed tomography, the proper indications and limitations of liver scanning deserve re-evaluation.

I. Historical Note

Hepatic scintigrams, using radioactive tracers to demonstrate space-occupying defects in the liver, were first reported by Stirrett et al (1) in 1954. The radioactive isotopes useful for liver scanning have changed over the years. Rose bengal iodine-131 (^{131}I), taken up by the hepatocytes, was administered in the earlier studies, but greater uniformity of uptake pattern in normal livers and sharper delineation of defects in abnormal livers led to the employment of radioactive gold (^{198}Au) in the 1960s. One valuable asset of colloidal gold is its retention by the Kupffer cells of the liver which permits repeated scans to be made if necessary. Its retention is independent of liver function. On the other hand, the clearance of the ^{131}I-rose bengal is a reflection of the hepatic function, and maximum hepatic concentrations occurred about 1 hour after administration. A repeated scan 2 hours later demonstrated the iodinated compound in the gallbladder, supplying an index regarding the function of that organ. By placing the collimator over the abdomen after the ingestion of a glass of cream or milk, one may determine the rapidity of excretion of the rose bengal via the common duct into the intestine. This method has proved to be valuable for patients with jaundice in the differentiation of parenchymal liver disease and extrahepatic obstruction.

In 1949, Dudley and Maddox (2) showed experimentally that gallium-72 (^{72}Ga), administered as citrate or lactate, was selectively localized in bone and proliferating tissues. In 1953, Andrews et al (3) found that not only bone tumors but some highly cellular soft tissue tumors as well had a fairly high concentrating capability of ^{72}Ga or gallium-67 (^{67}Ga). In 1969, Edwards and Hayes (4) showed that Hodgkin's lesions had a high uptake of ^{67}Ga. Among 25 patients with primary or metastatic cancers of the liver, Suzuki et al (5) found that gold scintigraphy detected a higher percentage of lesions than did gallium scintigraphy (76% versus 52%), and hepatic arteriography demonstrated cancers in 81% of the patients. There was a positive correlation between visualization of the lesion with gallium citrate and vascularity within the tumor. A gallium scan is often employed in localizing internal abscesses, lesions of melanoma, or malignant lymphoma where 50%–65% of biopsy-proven lesions were detected by the scan (6).

In the late 1960s, technetium-99 (^{99}Tc) sulfur colloid, taken up by both normal liver and spleen reticuloendothelial cells, was found to be superior to the radioactive gold because of improved counting statistics, reduced scanning time, lower radiation dose, and better spatial resolution. Approximately 80% of the technetium radioactivity is located in the liver, 10%–15% in the spleen, and 5% in the bone marrow (7). Haynie et al (8) compared the results using two different isotopes. The false-negative rate in abnormal livers with neoplastic or nonneoplastic disease was similar (20%), whereas in normal livers, technetium gave a lower false-positive rate than gold (11% versus 26%). In 48 patients with suspected liver metastases, Cantor et al (9) found that the technetium scan demonstrated approximately 10% more intrahepatic lesions, espe-

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cially those of malignant lymphomas. Thus, ^{99}Tc sulfur colloid has become the most widely utilized agent today.

II. Liver Scanning as Compared With Other Noninvasive Tests[3]

In 1963, Nagler et al (10) reported that liver scan reports could be correlated with histologic findings in 83% of 402 patients who had positive scans and in 88% of 146 patients with negative scans. For patients with focal lesions, only 72% had a correct diagnosis made by the liver scan. Ariel et al (11) found that liver scans correctly diagnosed the pathology in 85% of the patients who had laparotomy or necropsy. Castagna et al (12) found that scans correctly predicted the presence or absence of metastases in 74.3% of 109 patients. Alkaline phosphatase was increased in only 58% of Castagna's patients with proven liver metastases. Thingan et al (13) believed that alkaline phosphatase and BSP retention tests were as sensitive in detecting liver metastases as a scan but that they produced a somewhat higher false-positive rate.

Among patients with localized malignant and benign lesions, Gollin et al (14) found that abnormal rose bengal scans were highly reliable, because there was a false-positive rate of only 2.5%; however, normal scans were considerably less dependable, because there was a false-negative rate of 21%. They considered their overall accuracy rate of 77% to be much better than any other single liver function test, since alkaline phosphatase, which was almost as sensitive in detecting localized disease, yielded false-positives twice as often. Using both the radioisotopic scan and the alkaline phosphatase provided the most reliable index to the presence or absence of hepatic disease. Wilson et al (15) also showed an increase in accuracy from 70% to 92% by correlating both the liver scanning and alkaline phosphatase examinations.

On the other hand, Rosenthal and Kaufman (16) showed that the liver scan may be only an academic exercise. They correlated liver scan findings with physical examinations and routine laboratory tests in 111 patients with extrahepatic malignancies (39 of 111 had documented hepatic metastases). The liver scans were positive in 15 of the 16 patients with abnormal liver function tests and hepatomegaly. Of ten patients with liver metastases but normal laboratory tests and liver size, only one patient had a positive scan. Among 62 patients with documented absence of metastases, the scan was positive in three and equivocal in seven. In Rosenthal and Kaufman's study, the liver scan did not add significant information to that gained from the clinical and routine chemical assessment.

Sugarbaker et al (17) evaluated the accuracy rate of the liver scintiscan in combination with plasma carcinoembryonic antigen (CEA) assay or standard liver function tests for the detection of hepatic metastasis from carcinoma of the breast. In 55 patients, 47 with recurrent disease and 29 with documented hepatic metastasis, they found that, as a single test, the liver scan had a true-positive rate of 89% and a false-positive rate of 14%. Alkaline phosphatase, SGOT, and LDH each had a sensitivity rate of about 70% but a false-positive rate of 40%. An elevated CEA level (> 2.5 ng/ml) was slightly more sensitive than a liver function test (true false-positive rate = 76%) and it was also more specific (false-positive rate = 25%). When composite tests were formed by requiring both of the two individual tests to be positive, a positive liver scan and abnormal CEA level yielded 74% true-positive results with no false-positive outcomes. When a liver scan and CEA assay disagree, Sugarbaker et al suggested using other methods to improve diagnostic accuracy.

Rossi and Gould (18) found no significant difference in the reliability of the positive results between angiography and scanning (88% and 81%, respectively). But Almersjo et al (19) reported a very high false-positive rate (70%) for liver scanning, while the number of false-positive angiograms was far less (15%).

III. Liver Scanning as Compared With Invasive Tests

Ferrier et al (20) found that hepatic scintigraphy was more accurate than biochemical liver function tests or blind needle biopsy for the detection of hepatic metastases. In 100 patients with documented liver metastases, Maxfield et al (21) re-

[3] In this review, only results of definitely positive or abnormal scans will be considered as diagnostic of liver metastasis. Suspicious or equivocal scans are counted as negative. A false-negative report means patients had the disease or condition but the diagnosis was missed by the particular test. A low false-negative rate means that the test had high sensitivity. The false-negative rate is the proportion (or %) of patients who had documented disease among the subpopulation of patients who had normal scans. A false-positive report means patients were diagnosed to have the disease by the test, but actually did not have the particular disease. A low false-positive rate means the test was very specific. The false-positive rate is the proportion (or %) of patients known to be without disease among the subpopulation of patients who had positive test scans. The accuracy rate refers to how good a test can detect the absence or presence of a particular disease. Overall accuracy rate is equal to the sum of patients correctly diagnosed as having or not having the disease by the test divided by the total population tested.

ported that liver scans had an accuracy rate of 90% (false-positive and false-negative rates, each 5%). A needle biopsy of the liver was 92% accurate, with a false-negative rate of 8%. By contrast, alkaline phosphatase, SGOT, and BSP retention were about 75% accurate, with a false-negative rate of 23% and a false-positive rate of 2%. Bilirubin, albumin, and globulin were much less sensitive, with a false-negative rate of 40%–65%.

The relatively high incidence of false-positive results of liver scans for metastatic disease has prompted Sauer et al (22) to include peritoneoscopy for the evaluation of possible hepatic metastasis. Using this approach and combining it with needle biopsy, they reported 7% false-negative and no false-positive results. Bleiberg et al (23) compared liver function tests, liver scanning (with gold colloid), and peritoneoscopy in 240 patients with carcinoma: 44 had definite liver metastases. They had a more strict definition of positive liver function tests—an increase of 20% above the upper limit of normal range noted in two consecutive determinations. False-negative findings of liver chemistry tests, liver scan, and peritoneoscopy were seen in 27%, 42%, and 36%, respectively, while false-positive results were seen in 15%, 10%, and 3% of the patients, respectively.

There are three techniques useful in obtaining liver samples for histologic examination. Laparotomy would detect almost all metastases, but false-negative results, ranging from 5% to 16%, were also reported with this surgical staging. Percutaneous needle biopsy has been used for many years, and according to Conn and Yesner (24), the liver biopsy was 45% positive if ≥ 25 nodules were scattered throughout the liver, and substantially less with a lesser degree of involvement. In patients with suspected hepatic metastases from various origins, blind percutaneous biopsy followed by peritoneoscopy with a second biopsy under guidance have similarly been compared; the yield was 39.5% and 69%, respectively (25). Systematic peritoneoscopy with guided-needle biopsy is, at present, increasingly used for the diagnosis of metastasis to the liver. However, peritoneoscopy with biopsy of the liver has some important limitations. It must be carried out with caution, particularly after an abdominal operation, and cannot be endlessly repeated in the same patient. Even under favorable conditions, deep or posterior hepatic lesions cannot be observed. Peritoneoscopy also failed to provide an adequate tissue sample in a substantial number of patients, particularly in the presence of diffuse peritoneal adhesions (23).

IV. Explanation for Inaccuracies of Liver Scanning

Although hepatic scintigraphy is probably the most sensitive method for the early detection of hepatic metastasis, nevertheless, only 74%–90% of those with documented liver lesions had abnormal scans (26). Despite recent developments in radiopharmaceuticals and instrumentation, it has been found that the diagnostic accuracy of static liver scanning, from the days using gold colloid to recent periods using technetium sulfur colloid, has not improved (27). Several basic limitations exist.

The metastasis must be large before it can be detected on the hepatic scintigram. Thus, Friedell et al (28) and Wagner et al (29) found that the minimum size lesion that could be seen on the hepatic scintigram was 2–2.5 cm in diameter where the tissue was 10 cm thick. Where the tissue was only 5 cm thick, a lesion 1.5 cm in diameter could be seen. It has been suggested by others that a lesion must be at least 4.5 cm in diameter if it lies in the center of the right lobe, or 1.8 cm in diameter if it is peripheral, before it will be visualized on the scintigram (30).

Liver scanning is quite nonspecific. Motion artifacts and respiratory movement can cause irregularity of superior and inferior margins. Normal variants, such as the interlobar fissure, hepatic veins, major biliary radicals, and the gallbladder fossa, may mimic space-occupying lesions (31). Some conditions which produce abnormal liver scans include cirrhosis (pseudotumors), benign tumors, large kidneys, congenital cysts, liver abscess, dilated bile ducts, and enlarged nodes in the hilum (21,32). Technical difficulties such as field uniformity problems with the scintillation camera could result in abnormal focal defects on the scans (33). False-positive liver scans were noted in patients who had total colectomy, perhaps secondary to decreased portal blood flow and compensatory increased uptake in the spleen (34).

Although cone-down views, dynamic flow study, radionuclide tomography, and serial tests have been proposed to improve the accuracy of static views, the full cost-effectiveness of these additional studies remains to be determined.

Another difficulty influencing the reliability of liver scanning relates to subjective interpretation of the images. Liewendahl and Schauman (35), in a statistical analysis of 405 patients with focal defects in liver scans, believed this to be a highly reliable abnormal finding with a false-positive rate of 4%, and agreed that the findings of decreased activity were of limited value. Using strict scintigraphic criteria (focal defects only) for diagnosing hepatic

metastases in patients with breast cancer yields a true-positive rate of only 67% and a false-positive rate of 9% (36). The use of more liberal criteria for positive diagnosis (such as focal defects and heterogeneous distribution of radiocolloid or hepatomegaly) increased the true-positive rate to 89% and the false-positive rate to 14% (17).

Among 323 patients with various solid tumors, excluding breast cancers, Felix et al (37) reported that the overall accuracy of the liver scan based on focal defects was 95% with a 4% false-positive rate. When abnormal liver scans included enlargement, patchy or nonhomogenous uptake, but no focal lesion, the accuracy rate decreased to 83% and the false-positive rate increased to 16%.

Conn and Spencer (38) asked three nuclear physicians to examine 140 consecutive technically satisfactory liver scans, one third of which had previously been diagnosed as containing filling defects. The physicians were given no clinical information. Among the 140 scans, the inter-observer overall agreement was 70%. They agreed more often about the presence or absence of filling defects than about less discrete aspects of the scans, such as size, shape, position, homogeneity of uptake, etc. Ludbrook et al (39) asked seven observers to examine 99 liver scans for space-occupying lesions. On best performance, the average overall error rate was 20%. This degree of observer variability has been found in interpretations of chest roentgenograms, esophagrams, electrocardiograms, etc. In general, the inter-observer variation ranges from 20% to 25%, and the intra-observer error from 15% to 20% (38).

V. Liver Scanning as a Routine Initial Screening Procedure

As reviewed in previous sections and summarized in table 1, there are variations in the accuracy of the liver scan in patients with suspected or documented metastases: it ranged from 74% to 84% (false-positive rates were as low as 13% and as high as 42%,

TABLE 1.—Results of liver scintiscanning findings as correlated with histopathology

Investigator (ref No.) and yr	No. of patients	Agent*	% of patients with liver metastases	Method of confirmation	False-positive rate	False-negative rate	Overall accuracy	Remarks
Nagler et al (10), 1963	548	I-RB	64	Surgery, autopsy, and followup	17% (69/402)†	12% (18/146)†	84% (449/581)†	90% cancer patients
Castagna et al (12), 1972	109	I-HSA	49	Surgery and autopsy	28% (16/57)	23% (12/52)	74% (81/109)	Cancer, all sites
DuPriest et al (55), 1973	75	Tc-SC	33	Biopsy, surgery, and autopsy	33% (5/15)	21% (9/42)	74% (42/57)	Suspected liver metastasis
Rosenthal and Kaufman (16), 1973	111	Tc-SC	35	Biopsy or surgery	13% (3/24)	21% (18/87)	81% (90/111)	Cancer, all sites
Fee et al (33), 1974	70	Tc-SC	31	Surgery	26% (6/23)	11% (5/47)	84% (59/70)	Suspected abdominal tumors
Lunia et al (27), 1975	581	Tc-SC	44	Biopsy, surgery, and autopsy	17% (31/183)	25% (101/398)	77% (449/581)	Cancer hospital patients
Felix et al (37), 1976	32	Tc-SC	3	Surgery	67% (12/18)	2% (5/305)	95% (306/323)	Melanoma, sarcoma, head and neck, pelvic; early cancer
Bleiberg et al (23), 1977	59	Au	25	Peritoneoscopy and surgery	42% (8/19)	10% (4/40)	80% (47/59)	Cancer, all sites
Totals								
Range‡			25–64		13%–42%	10%–25%	74%–84%	
Overall‡					19% (138/723)	21% (167/812)	81% (1396/1731)	

*Au = radioactive gold; I-RB = iodine-rose bengal; I-HSA = iodine-human serum albumin; Tc-SC = technetium-sulfur colloid.

†Data are calculated according to the following definitions: false-positive = No. of false-positive tests/No. of positive tests in the series; false-negative = No. of false-negative tests/No. of negative tests in the series; and overall accuracy = proportion of patients correctly diagnosed as negative or positive in the series.

‡For calculation of range and overall rates, the series of Felix et al (ref 37), which was composed exclusively of patients with early operable solid cancers, was excluded.

while false-negative rates varied from 10% to 25%). Based on these data, many authors have suggested that the liver scan be used as a screening test in cancer patients. On the other hand, Rosenthal and Kaufman (16) have strongly argued against the routine use of liver scanning in cancer patients. In their study, the liver scan did not add significant information to that gained from the clinical and routine chemical assessment. Several other investigators have recently reported series which have also shown that liver scanning, although benign, is expensive and time consuming and has too many false-positive findings to be indicated in routine preoperative screening of patients with operable solid tumors.

A. Breast Carcinoma

Jasinski et al (40) found a 20% incidence of hepatic metastasis on routine hepatic scintigram in patients with mammary carcinoma admitted for radiotherapy. Sears et al (41) reviewed liver scans as part of the initial evaluation of 100 patients with potentially resectable carcinoma of the breast. Five positive scans were reported, and four of these were found to be false-positive. Another 154 liver scans were performed on 65 patients known to have recurrent, residual, or metastatic disease. Eight (12%) of 65 patients had 25 abnormal scans which correctly identified liver metastases. Four of these patients had palpable livers, and five had abnormal hepatic enzymes. Wiener and Sachs (42) reviewed routine perioperative liver scanning for patients with breast cancers. Only one of 231 patients considered to have normal livers had a positive scan. There were eight false-positive studies as established by surgical exploration, biopsy, and subsequent followup examination. Among 96 patients studied during the followup period, 24 had abnormal scans: 19 had documented liver metastasis (almost all had abnormal liver function tests) and five were false-positive (four had normal liver function tests). Based on their experience, they felt that the routine use of liver scans in evaluating the patients with carcinoma of the breast either preoperatively or postoperatively in the absence of abnormal liver function tests was not indicated.

B. Malignant Melanoma

In a large series of 600 melanoma patients treated over 6 years, Seigler and Fetter (43) found that the liver-spleen scan was helpful in only 2% of the scans done in patients without documented evidence of metastatic disease. However, the scan was positive 25% of the time if the patient's disease was widespread.

The experiences reported by other investigators were similar and showed that liver scans should be reserved for patients with specific symptoms or abnormalities. Among 73 patients who had primary melanoma or regional nodal disease, Roth et al (44) reported that none had abnormal findings on routine liver scans. But, among 22 patients with clinically disseminated diseases, about 18% had abnormal liver scans.

Among 99 patients with melanoma, one (3%) of 35 clinically stage I, four (7%) of 54 stage II, and four (36%) of 11 stage III patients had definite liver metastases. Felix et al (37) found that hepatic involvement was present in 71% of those patients with positive liver scans and in 35% of those patients with elevated liver chemistries (≥ 15% above normal range). All of the patients with positive scans and liver function tests had confirmed liver involvement with melanoma.

C. Lung Cancer

The routine use of liver scanning in 64 patients with lung cancer in whom liver metastases were not suspected resulted in only three definitely positive and five suspicious scans (45). Confirmation was eventually obtained in three of the latter scans, but methods by which diagnoses were verified were not described. With clinical suspicion of liver metastases (basis for suspicion not stated), the scan was positive in 13 (68%) of 19 patients. The overall incidence of positive scans in the patient population was 16 (19%) of 83 patients.

However, Hansen and Muggia (46) warned about accepting this high positive scan rate of 19%. They performed peritoneoscopy, routine liver scans, and function tests on 70 patients with inoperable lung cancer limited to the involved hemithorax. Six of the 16 patients with positive scans had subsequent documentation of liver metastases, whereas only four positive biopsies occurred in the 69 patients with suspicious or negative scans. They commented that if only patients with abnormal scans and all patients with small cell carcinoma had been selected for peritoneoscopy, liver metastasis would have been established in ten (32%) of 31 patients, a not inconsiderable yield. But a recent report showed that even in patients with small cell carcinoma of the lung, only one brain scan in 35 and only one liver scan in 21 yielded new information which suggested organ involvement (47). In patients with lung carcinoma who are incurable by surgery or radical radiotherapy, pretreatment brain and liver scans yield only 4% and 5% positive studies, respectively (45).

D. Other Solid Tumors

Fee et al (33) evaluated the value of preoperative

screening liver scanning in 70 patients before operation for suspected intra-abdominal tumors. The overall accuracy of the liver scan was 84% (26% false-positive and 11% false-negative results). For comparison, the accuracy of the serum alkaline phosphatase levels was 63% (13% false-negative and 56% false-positive results). The authors commented that of all the single diagnostic tools, the liver scan, inaccurate as it is, is probably still the best.

However, there are very few indications for performing preoperative screening liver scans in patients with suspected intra-abdominal tumors, because the liver can be easily examined at laparotomy and malignancy of the colon might require palliative resection even when hepatic metastases are present. In general, liver scanning in detecting metastases from gastrointestinal sites has an accuracy rate similar to the overall results reported (table 1). Among 129 patients with colorectal cancers, Ariel et al (11) reported that scans correctly diagnosed the findings in 86%, and Lunia et al (27) reported an accuracy rate of 85% in 118 patients. For carcinoma of the upper gastrointestinal tract (pancreas, stomach, etc), the reported accuracy rate was 70%–95%.

Felix et al (37) evaluated the value of routine preoperative liver scans in 224 patients with operable tumors, including 87 patients with head and neck cancers, 72 with sarcoma, and 65 with pelvic malignancies. Only one patient had liver metastasis identified by a liver scan; another patient with liver metastasis had a normal scan (both patients had advanced carcinoma of the cervix). In addition, 12 patients with positive liver scans had no evidence of hepatic metastasis within 1 year (false-positive rate, 67% [12 of 18]). They felt that, overall, the percentage of positive tests corresponding to metastatic disease in the liver was higher for the scans (33%) than for the liver chemistry battery (15%), but was significantly greater when the two examinations were considered simultaneously (83%). The liver scan was most useful as a back-up for the routine liver tests to confirm and localize the presence of liver metastases, and not as an initial procedure.

VI. Liver Scanning Compared With Ultrasonography and Computed Tomography (CT)

During the past few years, ultrasonography of the liver has been and is able to differentiate between cysts and solid tumors (48). It is not widely used, but with further technologic improvements it may become useful for screening the liver for metastases. Another more exciting development is the whole-body scanner of CT.

Since the first CT image of the liver in 1975 (49), remarkable technologic advancements have permitted CT to become a clinically valuable diagnostic method. With few exceptions, the radiographic density of most malignant tumors in the liver has been between that of water and normal liver. The tumors are most apparent in livers that have a relatively high normal parenchymal density which provides a natural contrast. Some tumors have very subtle density differences and require manipulation of window settings for detection; others can be detected only after administration of contrast material. Despite the variety of neoplasms studied, there is little evidence to suggest that the histologic type, site, or origin of a tumor can be predicted on the basis of CT appearance (50). The CT scan is particularly helpful in solving problems where there is conflicting information from other radiologic or laboratory studies.

Bryan et al (51) compared the ability of CT, gray-scale ultrasonography, and radionuclide image to detect and characterize space-occupying processes in the liver in 51 patients. The false-positive rates for these three tests were 0, 2%, and 4%, and the false-negative rates were 12%, 6%, and 4%, respectively. Motion artifacts and stellate artifacts from metallic surgical clips obscure CT images, while overlying bowel gas caused confusion for ultrasonography. They concluded that these three tests appeared to be complementary studies, each supplying some unique information not available from the others. Although radionuclide imaging scored the lowest overall rating in their study, its ease of performance, the ability to image all of the liver in all patients, and the low incidence of false-negatives make it the modality of choice for initial screening of the liver. The only exception to this is in patients with lymphoma, where ultrasonography is probably the screening procedure of choice.

When a definite or suspected abnormality is observed by nuclear imaging, either ultrasonography or CT should be performed to obtain better anatomic definition and characterization of the process. Both CT and ultrasonography were helpful in outlining nearby organs and structures such as the pancreas, spleen, kidneys, bladder, aorta, and paraaortic nodes. Since it costs less and does not involve the use of ionizing radiation, and also since it can scan the liver in more than one plane, ultrasonography is preferable to CT at the moment.

Schaner et al (52) performed whole-body CT in 600 patients with known or suspected malignancy. CT proved valuable in defining many pathologic processes and was, at times, deemed the only test short

of exploratory surgery that yielded diagnostic information. CT was most useful in lesions affecting the retroperitoneum, adrenal glands, pelvis, liver, and pulmonary parenchyma. Among 133 patients who had liver CT studies, five had malignant lesions. Lesions within the liver are usually less dense than the surrounding normal hepatic parenchyma. This difference can be accentuated by the iv infusion of iodine-containing contrast material. Liver cysts, solitary and multiple metastases, and primary hepatic neoplasms have all been well-demonstrated. CT is particularly valuable in identifying small lesions (down to 5 mm in diameter) deep within the hepatic substance and therefore not visible with nuclear scanning. However, a specific limitation in the detection of intrahepatic lesions by CT occurs with hypervascular masses occupying a significant portion of the liver substance. This principally includes large hepatomas and hypervascular metastases.

CT has been found to be of specific value in distinguishing medical from surgical jaundice (51,53). It provides a rapid and noninvasive means of visualizing dilated intrahepatic ducts and their radicles, and can be used in critically ill patients in whom more invasive procedures such as transhepatic cholangiography cannot be performed.

Intrahepatic lesions localized in CT scans can be biopsied using either a 23- or 14-gauge needle (54). The obvious advantages of this CT guidance technique are that one is better able to avoid other structures such as the diaphragm, costophrenic angles, colon, and gallbladder, and there is a higher probability of obtaining a positive specimen. Complications of bleeding from a percutaneous biopsy should be no different from those of the blind technique.

VII. Summary

The early diagnosis of hepatic metastasis is extremely difficult. In patients who had laparotomy for hepatic tumors, there was a rather high incidence of abnormal liver function tests in patients found to have normal livers, and a rather high incidence of normal test results in patients with liver tumors. For instance, alkaline phosphatase had a false-positive rate of 40%–56% (17,33) and a false-negative rate of > 10% (33). Hepatic scintigraphy, using radioisotopes taken up by the reticuloendothelial cells, is more sensitive and has an accuracy rate of about 74%–90% (table 1). False-positive rates have been reported as low as 13% and as high as 42%, and false-negative rates have varied from 10% to 25%. The accuracy of the liver scan could be increased if one correlates radioisotopic results with elevated alkaline phosphatase (14,42) or CEA levels (17).

There are several limitations to liver scintiscanning. The lesion must be > 2 cm (or 5 cm if it lies deep in the right lobe) to be visualized on the scintigram. Liver scanning is nonspecific; many normal variants or benign conditions can produce abnormal images. Technical difficulties and subjective interpretation of the scans also influence its reliability.

For patients with clinically localized extra-abdominal tumors (ie, breast carcinoma, head and neck carcinoma, malignant melanoma, and sarcoma), a routine liver scan is not indicated as part of the initial screening study, because true-positive scans were seen in 1% of the patients (37,41,44) and false-positive scans were seen in about 4% (37,41). In patients with recurrent or metastatic disease, approximately 12%–20% had positive scans (41,44). Despite the fact that many of such patients already had abnormal liver function tests or hepatomegaly (16), the liver scan is useful as a back-up to confirm and localize the presence of hepatic metastases and to offer an objective measurement of response to therapy.

Comparing liver scanning to newer techniques of ultrasonography and CT, Bryan et al (51) recommended using the hepatic scan as the initial modality of choice for diagnosing space-occupying lesions in the liver. When a definite or suspected abnormality is observed by nuclear imaging, either ultrasonography or CT should be performed to obtain better anatomic definition and characterization of the process. However, both ultrasonography and CT, like liver scanning, hepatic angiography, percutaneous needle biopsy, peritoneoscopy, and guided-needle biopsy, etc, have their limitations. The full cost-effectiveness of all of these studies, either singularly or in combination, remains to be determined.

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5-Year Survey of Methotrexate, Cyclophosphamide (Endoxan), and Vincristine (MEV) Therapy for Advanced Non-Hodgkin's Lymphomas [1,2]

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SUMMARY

Five courses of a combination chemotherapy regimen with methotrexate, cyclophosphamide (Endoxan), and vincristine were given to 64 patients, 37 with lymphocytic lymphoma (LL) and 27 with histiocytic lymphoma (HL). Eighteen of the 37 patients with LL (48%) achieved a complete remission (CR); 12 of these 18 patients have been in CR for 20–58 months. Among the complete responders only two died. Twenty-two of the 27 patients with HL (81%) achieved CR and 12 of these patients are still in CR. Ten of the complete responders have been relapse-free for 24–70 months and five have been relapse-free for > 60 months. Clinical and hematologic tolerance was such that the interval between each course had to be prolonged in only three of the 64 patients and, with the exception of vincristine, the dosages were not reduced.

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Non-Hodgkin's lymphomas are a heterogeneous group of diseases. Their management remains controversial and unsatisfactory in spite of the impressive number of therapeutic regimens and systems of histologic and immunologic classifications (1,2) that have been developed in the last few years.

Recently, the role of radiotherapy has become more limited, while polychemotherapy is currently agreed on as a major tool for the treatment of stage III and IV non-Hodgkin's lymphomas (1,3,4).

In 1975 (5), we demonstrated that a combination of methotrexate, cyclophosphamide (Endoxan), and vincristine (MEV regimen) could induce remission in histiocytic lymphoma (HL). This study was based on a limited series of 30 patients with a followup ranging between 5 and 36 months. We now report on a series of 64 patients treated with MEV, with a followup ranging between 12 and 70 months. We wish to emphasize the high level of efficacy and the low toxicity of this regimen.

MATERIALS AND METHODS

From September 1971 to December 1975, 67 untreated patients with advanced non-Hodgkin's lymphoma were admitted to the Division of Hematology, St. Orsola's University Hospital, Bologna, Italy. The tumors were classified histologically using the Rappaport system (6). Three patients with mixed (lymphocytic-histiocytic) subtype were excluded and the remaining 64 patients were entered in the MEV regimen.

Patients were staged in a manner similar to that used for Hodgkin's disease (7), including lymphangiography and bone marrow biopsy. Laparotomy and splenectomy were carried out on 21 patients. Of the remaining 43 patients, 14 were determined to be stage IV by bone marrow and/or liver biopsy and the other 29 were found not eligible for laparotomy because of advanced age (> 60 years) or medical contraindications (diabetes mellitus, lung or heart disease, or impaired renal function). All patients received five consecutive courses, at intervals of 15 days, of cyclophosphamide (800 mg/m^2 in a 4-hour

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TABLE 1.—Patient characteristics (MEV regimen)

	LL	HL	Totals
Total No. of patients	37	27	64
Median age in yrs (range)	51 (10–78)	54 (16–83)	52 (10–83)
Sex (males/females)	22/15	15/12	37/27
Clinical variety (A/B)	30/7	18/9	48/16
Stage			
I	—	3	3
II	1	5	6
III	11	13	24
IV	25	6	31

TABLE 2.—Patient distribution according to histology

Histology		No. of patients
LL		
Nodular well-differentiated		4
Nodular poorly differentiated		11
Diffuse well-differentiated		6
Diffuse poorly differentiated		16
	Total	37
HL		
Nodular		8
Diffuse		19
	Total	27

infusion on Day 1), methotrexate (20 mg/m² im on Day 3), and vincristine (2 mg/m² iv on Day 4). Blood counts were repeated at the beginning of every cycle.

Patient data are reported in tables 1 and 2. Thirty-seven patients with previously untreated lymphocytic lymphoma (LL) and 27 patients with previously untreated HL had completed MEV therapy by April 1976. The majority of patients had diffuse lymphomas. Nine patients with stage I and II were considered ineligible for radical radiotherapy because of advanced age (> 60 years). However, four of these patients who had localized supradiaphragmatic disease also received local radiotherapy; their stage and histology were as follows: IIA lymphocytic nodular well-differentiated, IIA histiocytic diffuse, IA histiocytic diffuse, and IA histiocytic nodular. Two patients with HL (stage IIIB diffuse type and stage IIA nodular type) and one patient with LL (stage IIIA diffuse poorly differentiated type) were given total nodal irradiation after MEV-induced complete response (CR). Neither maintenance nor reinduction therapy was given to any of the other patients who achieved CR. CR was defined as the absence of any clinical, radiologic, or biochemical evidence of lymphoma and was checked by physical examination, radiograms, and laboratory investigations. Lymphangiography and liver biopsy were never repeated. Bone marrow biopsy was repeated when it was positive before therapy. Remission and survival durations were calculated from the end of treatment. The method described by Kaplan and Meier (8) was used for the survival analysis.

RESULTS

CR was achieved in 18 of 37 patients (48%) with LL (table 3). The remission rate of 60% (nine of 15) in patients with nodular LL was somewhat higher compared to the remission rate of 41% (nine of 22) in patients with the diffuse histologic subtype. The remission rate of 54% (six of 11) in patients whose disease was limited to the lymph nodes (stage I–III) was also somewhat higher compared to the remission rate of 41% (12 of 29) in stage IV patients. CR was obtained in patients without general symptoms (type A disease) while none of the six patients with general symptoms had CR. Twelve of the patients who achieved CR were relapse-free after 24–58 months. These 12 patients accounted for 66% of the patients who had CR and 32% of all LL patients who were entered in this study. Six patients (table 4) relapsed after 3–32 months and two of them died (two of the six relapsed patients had also received radiotherapy).

In patients with HL (table 3), the CR rate was very high (81%, 22 of 27 patients), without any apparent difference according to stage, type A or B, and histology. Twelve of the patients who achieved CR were relapse-free after 24–70 months. These 12 patients accounted for 54% of the patients who had CR and 44% of all HL patients who were entered in this study. Ten patients (table 4) relapsed after 3–17 months and two of them died (one of the ten relapsed patients had also received radiotherapy). The remaining five patients who failed to achieve CR died.

Actuarial survival and relapse-free survival are shown in figure 1. Median survival is 42 months for LL patients and has not yet been reached for HL patients. The prospective 5-year relapse-free survival approaches 30% for LL patients and 50% for HL patients.

Hematologic toxicity was negligible. All patients received five or six courses of MEV and the interval between each course had to be prolonged in only three of 64 patients. Mild neutropenia (1000–1500 granulocytes/μl) was observed after only nine of 284

TABLE 3.—CR rate according to histology and stage

Histology	No. of CRs/No. of patients (Stage) IA, IIA	IIIA	IIIB	IVA	IVB	Totals
LL						
Nodular well-differentiated	—	0/1	—	3/3	—	3/4
Nodular poorly differentiated	1/1	2/4	—	3/5	0/1	6/11
Diffuse well-differentiated	—	0/1	—	2/4	0/1	2/6
Diffuse poorly differentiated	—	3/3	0/1	4/9	0/3	7/16
Totals	1/1	5/9	0/1	12/21	0/5	18/37
HL						
Nodular	3/4	—	0/1	2/2	1/1	6/8
Diffuse	4/4	6/7	2/3	—	4/5	16/19
Totals	7/8	6/7	2/4	2/2	5/6	22/27

TABLE 4.—Frequency of relapse according to histology and stage

Histology	No. of relapsed patients/No. of CR patients (Stage) IA, IIA	IIIA	IIIB	IVA	IVB	Totals
LL						
Nodular well-differentiated	—	—	—	2/3	—	2/3
Nodular poorly differentiated	0/1	1/2	—	1/3	—	2/6
Diffuse well-differentiated	—	—	—	0/2	—	0/2
Diffuse poorly differentiated	—	1/3	—	1/4	—	22/7
Totals	0/1	2/5	—	4/12	—	6/18
HL						
Nodular	0/3	—	—	2/2	0/1	2/6
Diffuse	2/4	4/6	0/2	—	2/4	8/16
Totals	2/7	4/6	0/2	2/2	2/5	10/22

courses of MEV, and promptly recovered. Thrombocytopenia with a platelet count $< 100{,}000/\mu l$ was never recorded and the hemoglobin level did not fall significantly throughout therapy. Cyclophosphamide and methotrexate were always given at full doses without major subjective toxicities, apart from occasional nausea, malaise, and, more rarely, vomiting after cyclophosphamide. However, vincristine was reduced to a total dose of 2 mg in nearly all patients after the second dose and occasionally was discontinued because of developing neurotoxicity. Lymphocyte and immunoglobulin levels remained constant and normal in all patients, none of whom developed bacterial, viral, or fungal infections.

DISCUSSION

The MEV regimen is effective for remission induction in patients with HL. The initial CR rate of 81% favorably compares with the results of recent trials (4,9–11). However, ten of the 22 CR patients (seven of whom had a diffuse histologic subtype) relapsed for a long-term CR rate of 44% (12 of 27 patients). All patients relapsing did so within 17 months and this suggests that further chemotherapy given early after remission induction might have reduced the relapse rate. Notwithstanding the frequency of the relapses, the 2-year survival of all patients who had CR was > 90% and the projected 5-year survival approaches 90%. This suggests that MEV significantly prolonged survival of HL patients, very frequently inducing CR and allowing patients to be effectively treated after relapse.

In LL patients MEV was not as effective as in patients with HL and it was possibly less active than other regimens (12,13). In particular, MEV cannot be recommended for LL patients with type B disease, none of whom achieved a CR. In many patients, partial resistance to the MEV regimen may have been due to rapid regrowth of the lymphoma in the interval between the courses. This may suggest that the disease was sensitive to the

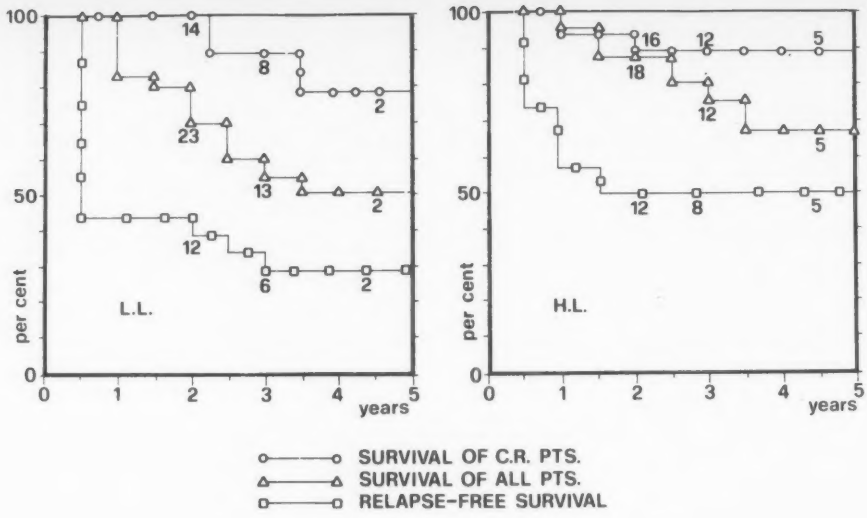

FIGURE 1.—Survival and relapse-free survival of the 64 non-Hodgkin's lymphoma patients treated with the MEV regimen.

drugs used, but the schedule was not optimal. However, it should be pointed out that 12 of the 37 patients (32%) were relapse-free after 24–58 months. There were no differences in stage and histology between LL patients responding to MEV and those who did not.

In summary, this 5-year experience with MEV in 64 patients demonstrated that the regimen was effective with minimal toxicity which allows outpatient management of advanced non-Hodgkin's lymphoma patients. Moreover, MEV was not immunosuppressive at a clinically measurable extent and did not induce lymphocytopenia or hypogammaglobulinemia.

Recently, the Stanford group (14) suggested that, for stage IV patients with favorable histology (nodular well-differentiated lymphocytic, nodular poorly differentiated lymphocytic, or diffuse well-differentiated lymphocytic), single therapeutic agents were as effective, in terms of survival, as the more aggressive kinds of treatment (cyclophosphamide, vincristine, and prednisone, used alone or with a split course of radiotherapy). Clinical and hematologic toxicity induced by MEV was also mild and this regimen had the potential of offering a long disease-free survival in a considerable number of patients. Therefore, it appears that today MEV may play an important role in the management of some forms of non-Hodgkin's lymphomas.

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Phase II Study of Dianhydrogalactitol in Malignant Glioma [1]

Brief Reports and Preliminary Communications

Pilar Espana, Peter H. Wiernik,* and Michael D. Walker [2]

Dianhydrogalactitol (DAG) is a new chemotherapeutic agent found to be the most active drug against the murine intracranial ependymoblastoma (1). DAG is the diepoxide of dibromodulcitol following dibromonation (2); it is soluble in water or propylene glycol and stable in aqueous solution for up to 8 hours. Although the compound is thought to be an alkylating agent on the basis of chemical reactivity, as well as cross-resistant to other alkylating agents (3), its mode of action may be the inhibition of DNA synthesis and, to a lesser extent, RNA and protein synthesis. It is a non-cell-cycle specific agent (4) and the most potent of all the dihalohexitols evaluated (5). DAG entrance into the cerebrospinal fluid is rapid, and drug accumulation in brain tumors is higher than in normal white matter (6). The drug is slowly eliminated from the cerebrospinal fluid, where its half-life is 20 hours. Approximately 30% of administered DAG enters the brain tissue compartment, which makes this drug promising for therapeutic application.

The purpose of this study was to evaluate the use of DAG in the treatment of patients with malignant astrocytoma.

MATERIALS AND METHODS

Seventeen patients with biopsy-proven malignant astrocytoma were treated at the Baltimore Cancer Research Center from July 1975 to July 1976. Three patients were excluded from this study: two who received a single dose of DAG and another who was lost to followup 6 weeks after the start of chemotherapy. Of the 14 evaluable patients, nine had grade IV astrocytoma and five had grade III astrocytoma. All patients underwent surgery with partial or subtotal resection of the tumor and all of them received radiation therapy to the whole brain at a total dose of 6000 rads. Steroids were given to all except one of the patients following surgery. Steroid doses were not increased unless clinical deterioration occurred. If increased doses of steroids were necessary, the patient was considered to have had a DAG treatment failure.

Eight patients received DAG as an adjuvant to surgery and radiation therapy. Treatment was started in this group a median of 5 weeks after surgery (range, 3–9 weeks). Four patients received DAG after prior therapy with methyl-CCNU (meCCNU) plus VM-26. Two of these patients achieved responses and two did not; response durations and the duration of nonresponding survival to these agents were not included in survival analysis. Two additional patients received DAG only after tumor regrowth following surgery and radiation. For all patients, duration of response and survival data were analyzed from the start of DAG therapy.

The median age of the patients was 57 years (range, 29–72 years). A computerized axial tomography (CAT) scan was obtained in every patient at the beginning of the study and periodically during the disease. Routine studies included cbc count, SMA-12 profile, radiograms, and gamma brain scans in addition to clinical evaluations for evidence of response. Patients were considered treatment failures if, after two treatments, there were signs and symptoms of progressive disease. A subjective response was defined as definite evidence of significant neurologic improvement for at least 1 month, as judged by at least two physicians, without relation to increased steroids. Objective response was defined as definite reduction of tumor volume on the CAT scan.

DAG was given at a dose of 150 mg/m^2 iv over approximately a 1-hour period with re-treatment at 21-day intervals. Patients who had significant marrow suppression had their dose reduced to 130 mg/m^2 and patients who had minimal or no toxic effects had the DAG dose escalated to 170 mg/m^2.

RESULTS

Fourteen of 17 patients with glioblastoma multiforme treated with DAG were evaluable for response. Nine of the 14 patients had subjective

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response to DAG and five had definite progression of disease. The median duration of response to DAG was 17 weeks (range, 8–74 weeks).

The longest survivals were seen among patients previously treated with meCCNU and VM-26. A 4-year survival was achieved in a patient who had a 7-month response to meCCNU-VM-26 followed by a 6-month response to DAG. Two other patients achieved 2-year survivals. One of these patients had failed meCCNU-VM-26 prior to a 19-month survival while receiving DAG, and the other did not respond to DAG after failing meCCNU-VM-26 chemotherapy.

The median survival of all DAG-treated patients was 72 weeks (range, 19–208 weeks) from diagnosis (surgical procedure) and 28.5 weeks (range, 15–79 weeks) from the start of DAG treatment. The median survival of patients with subjective response to DAG was 82 weeks (range, 30–208 weeks) from the diagnosis and 31 weeks (range, 25–79 weeks) from the start of DAG. For the group without clinical improvement, the median survivals were 47 weeks (range, 19–103 weeks) and 16 weeks (range, 10–67 weeks), respectively. The data for responders and nonresponders are not significantly different.

Toxicity

Leukopenia occurred in eight patients after treatment with DAG; on no occasion was it necessary to discontinue the drug. The median wbc count nadir of the eight patients was 2200/mm^3 (range, 800–2900/mm^3); the nadir was reached on the 16th day (range, 14–20 days) of treatment. Thrombocytopenia of $< 100{,}000$ platelets/mm^3 occurred in six patients. The median platelet count was 65,000/mm^3 (range, 34,000–86,000/mm^3); the nadir was reached on the 14th day (range, 7–18 days).

Other complications seen in our patients were: a single case of cellulitis at the site of DAG administration requiring skin graft, and four instances of stomatitis, nausea, and vomiting.

DISCUSSION

The median survival of 72 weeks from the diagnosis and 28.5 weeks from the start of chemotherapy is not significantly different from those survivals attained to date with other therapy (7–13). However, it should be noted that the patients in our group were relatively old, with only one patient < 30 years old (29 years), and most investigators feel that young people respond better (9,14).

Bone marrow toxic effects of DAG in this study were moderate and entirely acceptable. Only three patients required dose reductions but for three additional patients the drug was delayed 1 or 2 weeks due to marrow suppression.

DAG may lack cross-resistance with nitrosoureas. Three patients previously treated with meCCNU failed that treatment but responded to DAG. Response duration in those patients was 4, 6.5, and 18 months.

Toxicity with DAG in this study was less than that reported in other studies with nitrosoureas. Randomized studies with DAG and nitrosoureas may serve to more clearly define the relative merits of each in the treatment of malignant glioma.

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Phase II Study of the Failure of Vincristine and Bleomycin for Previously Treated Children With Metastatic Neuroblastoma: A Report From the Childrens Cancer Study Group[1]

Brief Reports and Preliminary Communications

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SUMMARY

Twenty-two patients with previously treated but progressive stage IV neuroblastoma received combination therapy with vincristine (1.0 mg/m²) followed in 6 hours by bleomycin (15 mg/m²). Therapy was administered twice weekly. No responses were seen in the 19 patients who received four or more doses of the bleomycin-vincristine combination.

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[2]This study was conducted by the Childrens Cancer Study Group (CCSG) and was supported by Public Health Service grants (CA) from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare. The following institutions and investigators participated in the study: University of Southern California, Los Angeles (*G. D. Hammond, J. Weiner, R. Honour,* and *H. Sather;* CA-13539); University of Michigan, Ann Arbor (*R. Heyn;* CA-02971); Children's Hospital of the District of Columbia (*S. Leikin;* CA-03888); Cornell Medical Center, New York, NY (*D. R. Miller;* CA-14557); Children's Hospital of Los Angeles, Calif (*G. Higgins* and *R. Hittle;* CA-02649); Babies Hospital, New York, NY (*J. Wolff;* CA-03526); Children's Hospital of Pittsburgh, Pa (*V. Albo;* CA-07439); Children's Hospital of Columbus, Ohio (*W. Newton;* CA-03750); Children's Orthopedic Hospital, Seattle, Wash (*R. Chard* and *I. D. Bernstein;* CA-10382); University of Wisconsin (*N. Shahidi;* CA-05436); University of Minnesota, Minneapolis (*M. Nesbit;* CA-07306); Children's Memorial Hospital, Chicago, Ill (*G. Honig;* CA-07431); University of Utah Medical Center, Salt Lake City (*E. Lahey;* CA-10198); Princess Margaret Hospital, Toronto, Canada (*M. Sonley;* Ontario Cancer Treatment and Research Foundation, grant 41–17); University of Rochester, NY (*M. Klemperer;* CA-11174); Children's Hospital of Louisville, Ky (*D. R. Kmetz*); University of British Columbia, Vancouver (*M. Teasdale;* Vancouver Foundation); Children's Hospital of Philadelphia, Pa (*A. E. Evans;* CA-11796); Indiana University, Indianapolis (*R. Baehner;* CA-13809); New Jersey College of Medicine, Newark (*L. Vitale;* CA-12637); Harbor General Hospital, Torrance, Calif (*J. Finklestein;* CA-14560); University of California, San Francisco (*A. Ablin;* CA-17829); and Rainbow Babies and Children's Hospital, Cleveland, Ohio (*S. Gross*).

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The poor overall survival of children with metastatic neuroblastoma has not improved appreciably in the last 10–15 years (1,2). A number of drugs active in this disease have been identified, including cyclophosphamide, adriamycin, vincristine, and DTIC, which in combination show increased responses (2–4). Since most of these drugs are highly myelosuppressive, we sought an agent which would show activity against the tumor but was not myelosuppressive. Bleomycin is a drug known to minimally depress the bone marrow, but it has not been evaluated in patients with neuroblastoma. The CCSG therefore evaluated the activity of bleomycin for children with previously treated metastatic (stage IV) (5) neuroblastoma.

Bleomycin is an antibiotic isolated from a strain of *Streptomyces verticillus.* It has shown activity against a variety of transplantable mouse tumor systems. Significant response rates were also achieved with a variety of human tumors, including squamous cell carcinomas of various sites, lymphoma, and testicular carcinoma (6).

In the present study, bleomycin was administered 6 hours following a dose of vincristine given to enhance the likelihood of an effect, based on the report of Livingston et al (7) suggesting that this combination is superior to bleomycin used alone. This combination was based on the concept that bleomycin may be most effective when cells are in mitosis and in the G_2 phase, and that vincristine can increase the number of cells in mitosis.

MATERIALS AND METHODS

Patients

Patients were < 16 years old and had metastatic neuroblastoma. Tumor type was verified either histologically or by demonstration of clumps of tumor cells in the bone marrow in combination with elevated urine catecholamines, vanillylmandelic acid (VMA), or homovanillic acid (HVA). Patients were resistant to conventional chemotherapy (including

cyclophosphamide and vincristine) and had a measurable lesion(s) (bone marrow involvement with tumor cells was considered measurable disease). Informed consent was required from the parents or legal guardians prior to entry into this study. Patients with stage IV_S disease were excluded.

Drug Program

Patients were given vincristine (1.0 mg/m²) and bleomycin (15 mg/m²) iv twice each week for a period of up to 6 weeks. Vincristine was given 6 hours prior to the administration of bleomycin. Drugs were obtained from the Division of Cancer Treatment, NCI.

During the course of treatment, hemoglobin concentration and wbc and platelet counts were determined at least every other week; changes in physical examination and measurable disease were recorded at least every 3 weeks. Bone marrow aspirations were performed every 6 weeks, as were urinary catecholamines if originally elevated. A reduction in the bleomycin dose was made if severe skin toxic effects and/or stomatitis was observed and the drug was to be stopped if pulmonary toxic effects were observed. Patients exhibiting toxic reactions attributable to vincristine were to receive bleomycin alone. Surgery and local X-ray therapy were permitted except, if possible, for the indicator lesion.

A minimum of four doses of bleomycin was required for a patient to be considered evaluable. This study was designed to analyze only complete remissions (CRs) and partial remissions (PRs). A CR was defined as the disappearance of all evidence of disease for ≥ 28 days; a PR was defined as a $> 50\%$ decrease in the size of all measurable lesions for ≥ 28 days. The bone marrow was required to establish a response for that index lesion.

RESULTS

Twenty-two patients were entered in the study and 19 received four or more doses of bleomycin. Eleven of the 19 patients had six or more doses of bleomycin, while the remaining eight had either four or five doses.

Toxicity was minimal. One patient experienced stomatitis which was attributed to bleomycin and the drug was withheld; that same patient experienced bone pain attributed to vincristine which was also withheld. There was no other reduction in drug dose. Two other patients had a moderate fever following bleomycin injection. Severe hyperpyrexia, as noted in patients with lymphoma receiving bleomycin, was not observed (6). Hematologic toxic effects were not observed.

DISCUSSION

This study was one of a series conducted by the CCSG to define drugs with activity against neuroblastoma. Agents identified to be active for patients with late-stage neuroblastoma are being incorporated into regimens for newly diagnosed patients with metastatic disease. Bleomycin was of particular interest since it is not suppressive to the bone marrow. Thus, it was particularly disappointing that bleomycin failed to show activity in this study. Although patients with less advanced disease treated for longer periods of time might have responded to this regimen, the results of this study suggest that clinically significant effects of this combination would be highly unlikely. Since significant drug-related toxic effects were not observed, it may be that bleomycin administered in greater amounts or by different dose schedules could prove effective.

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Phase II Trial of a Combination of Cyclophosphamide, Vincristine, and Methotrexate in Advanced Colorectal Carcinoma [1,2]

Brief Reports and Preliminary Communications

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SUMMARY

Twenty patients with advanced colon cancer were treated with a combination of low-dose weekly cyclophosphamide, vincristine, and methotrexate (COM) after demonstrated progression of disease with 5-fluorouracil (5-FU). Four patients (20%) had partial responses with a median survival of 15 months, six (30%) had disease stabilization for 3–16 months (median survival, 8 months), and ten progressed with a median survival of 5.5 months. All four partial responses and five of six stabilizations occurred in patients with a performance status of 0 or 1. COM appeared well-tolerated and at least as effective as alternative secondary chemotherapy following 5-FU failure.

[Cancer Treat Rep 62:1203–1205, 1978]

Chemotherapy trials in colon cancer patients who fail to respond to or relapse with 5-fluorouracil (5-FU) have generally given disappointing results. Drugs which give favorable responses if used as initial treatment are generally less effective if used after 5-FU (1). The duration of survival following failure to respond to or relapse with 5-FU has been reported to be as short as 2 months, so that secondary chemotherapy of colon cancer has generally been unrewarding (1).

We report here on a drug combination used as secondary chemotherapy (COM: cyclophosphamide, vincristine, and methotrexate) in colon cancer; it was well-tolerated and had suggestive activity. Cyclophosphamide was selected for inclusion in the combination based on two partial responses (PR) in a series of 25 colon cancer patients (2). Methotrexate was included based on a 15% response rate in a collected series of 128 patients (3) and a 5% response rate in a smaller series with stricter criteria of response (2). Although vincristine has little demonstrated activity in colon cancer, it was included because it was thought that it may increase the effectiveness of other anticancer drugs (4).

METHODS

Criteria for protocol entry included prior treatment with 5-FU, a performance status of ≥ 3 (capable of self care and not totally confined to bed) by the Eastern Cooperative Oncology Group criteria, and progressive disease demonstrated by the development of new lesions or the enlargement of preexisting ones.

Patients were followed with physical examination and cbc and platelet count estimates weekly for at least 6 weeks and then biweekly. SMA screening of liver enzymes and renal function, carcinoembryonic antigen quantitation, and chest roentgenogram and liver scan evaluation of affected areas were performed at least every 2 months.

Cyclophosphamide at a dose of 300 mg/m^2 iv, vincristine at a dose of 2 mg iv (initial dose) followed by 1 mg iv in subsequent treatments, and methotrexate at a dose of 20 mg/m^2 iv were administered once weekly. A full dose was administered provided the wbc count was ≥ 3000/mm^3 and the platelet count was $\geq 75{,}000$/mm^3. If the blood cell counts were below these values, treatment was delayed until recovery and then administered every 2 weeks. Poor systemic tolerance also led to extending the interval between treatments to every 2 weeks.

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Measurable disease consisted of any tumors that were measurable in at least one direction or hepatomegaly that extended ≥ 5 cm below the costal margin. Evaluable disease included liver scan evidence of filling defects. A complete response was defined as complete disappearance of all objective evidence of tumor with the development of no new lesions. A PR was defined as a 50% reduction in the sum of products of the largest diameter and its perpendicular diameter of all bidirectional measurable lesions or a 30% reduction in the sum of all unidirectional measurable lesions. Complete or almost complete clearing of liver scan defects qualified as a PR. A 25% increase in the size of existent lesions or the development of new lesions was defined as disease progression. Any lesser degrees of change were classified as stable disease.

RESULTS

Twenty patients were entered in the study between February 1974 and June 1977. All patients had received 5-FU: 17 as the only agent, one in combination with methyl-CCNU and vincristine, and two as a 5-fluoro-2′-deoxyuridine intrahepatic infusion. There were 11 women and nine men with ages ranging from 36 to 78 years (median, 64.5 years). Although all patients had evidence of disease progression at the start of COM therapy, two patients could not be classified as 5-FU failures: one because she refused further 5-FU after three weekly injections and one because cerebellar ataxia required cessation of 5-FU therapy. All other patients had received a dose of 600 mg/m² iv weekly for at least 6 weeks with demonstrated progression as defined above.

Of the 20 patients evaluated, four (20%) had a PR for 4–44 months (median, 6.5 months) and an additional six (30%) achieved stabilization of disease for 3–16 months (median, 4.5 months). Ten patients had progression. One patient who was classified as "stable" refused further treatment after 3 months because of poor tolerance (the same patient had stopped 5-FU after only 3 weeks of treatment). She died 14 months from the start of COM and is considered as having stabilization of disease for 3 months in this analysis. There were no complete responders.

One patient continues in PR for 44 months after diagnosis of hepatic metastases at surgery in April 1974. She initially received 5-FU at a dose of 600 mg/m² iv weekly, but after 6 months showed increasing disease on a liver scan concomitant with the development of palpable hepatomegaly and left supraclavicular adenopathy. She was changed to COM with subsequent complete regression of adenopathy and hepatomegaly. The liver scan improved, but has not completely cleared. After 18 months, COM was discontinued by another physician and the patient has been receiving maintenance therapy with oral 5-FU, 500 mg weekly, without further change in her status.

Two of the other partial responders had measurable improvement in hepatomegaly for 4 and 9 months, respectively. One had shrinkage of supraclavicular and paratracheal lymphadenopathy continuing for 4 months, but then moved to another city where she received other therapy.

The median duration of survival from the start of COM therapy was 8 months for all patients. The two partial responders who have died survived for 10 and 15 months, respectively. Those who had stable disease lived for a median of 8 months (range, 7–21 months). Patients who had progression lived for a median of 5.5 months (range, 11 days to 16 months). The one patient who lived for 16 months was the only patient in this series who responded to third-line chemotherapy (methyl-CCNU plus streptozotocin). The duration of survival of the ten patients who obtained a PR or stabilization of disease was significantly better than that of those who had progression ($P < 0.02$).

The initial performance status of patients was correlated with response to treatment. Only one of six patients with a performance level of 2 or 3 had evidence of antineoplastic effect (stabilization) with a 40% reduction in measurable tumor size for 3 months. Nine of 14 patients with a performance status of 0 or 1 had a PR or stabilization of disease (not significantly different).

Hematologic toxic effects were usually mild, reflecting the low dose of the potentially myelosuppressive drugs. Generalized fatigue that was distinguishable from vincristine-induced peripheral neurotoxicity was an unexpected dose-limiting side effect. This usually restricted weekly administration of COM chemotherapy to the initial 6–8 weeks. The peaking of hematologic toxic effects and constitutional symptoms at approximately 6 weeks resulted in the practice of instituting biweekly therapy after 6 weeks in most patients.

DISCUSSION

A low dose of weekly COM chemotherapy has demonstrated activity in 5-FU-resistant colorectal cancer. In 20 patients treated, 20% had PRs with an additional 30% achieving lesser benefit for at least 3 months. The duration of survival was also prolonged over that anticipated from previous reports of 5-FU-resistant colon cancer. The median

survival of all patients was 8 months with patients who achieved a PR having a median survival of at least 15 months. A significant improvement in survival was noted in those patients who achieved at least stabilization of their disease over those who had progression.

In part, these results may be related to the good performance status of most patients. Of six patients with a performance status of 2 or 3, only one had evidence of antineoplastic effect whereas all four of the patients with PR and five of the six patients with stable disease had a performance level of 0 or 1. Although these differences are not significant with this small sample size, they parallel the higher response rate seen with 5-FU in colorectal cancer patients with good performance status (5). The suggestion of patient benefit and limited toxicity in this small series of patients justifies further evaluation of this combination in previously treated colon cancer patients. Because of the lack of evidence of increased benefit from the addition of these drugs to 5-FU in other series (6,7), it is not likely that COM will increase the effectiveness of 5-FU if used simultaneously.

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cis-Dichlorodiammineplatinum(II) Alone Followed by Adriamycin Plus Cyclophosphamide at Progression Versus *cis*-Dichlorodiammineplatinum(II), Adriamycin, and Cyclophosphamide in Combination for Adenocarcinoma of the Lung [1,2]

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SUMMARY

Forty-one patients with proven metastatic adenocarcinoma of the lung were randomized into two comparable groups after stratification for performance status and presence of measurable or evaluable disease. Treatment 1 consisted of *cis*-dichlorodiammineplatinum(II) (P) at a dose of 15 mg/m²/day by iv push on Days 1–5 every 4 weeks. Upon progression or the onset of renal toxic effects, patients were crossed over to cyclophosphamide (C) at a dose of 400 mg/m², and adriamycin (A) at a dose of 40 mg/m², both given by iv push on Day 1 every 4 weeks. Treatment 2 (CAP-I) consisted of C-A in the same doses as above plus concurrent P, 40 mg/m² by iv push on Day 1 every 4 weeks. Eight patients receiving P developed serum creatinine values ≥ 1.5 mg/100 ml versus one patient receiving CAP-I ($P = 0.05$). Objective regressions occurred in two of 22 patients with P, five of 17 with C-A, and eight of 19 with CAP-I ($P = 0.025$; CAP-I versus P). There was a significant increase in the median duration of regression in patients responding to C-A following P (267 days) versus patients responding to CAP-I (95 days). The improved rate of response with CAP-I and the prolonged duration of response with C-A following P suggest a potentiating effect between P and C-A whether given simultaneously or sequentially.

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cis-Dichlorodiammineplatinum(II) (P) is a platinum coordination complex with activity against a wide variety of human neoplasms and a potential for producing both reversible and permanent nephrotoxic effects (1). Animal studies have demonstrated synergism between P and a variety of other chemotherapeutic agents including cyclophosphamide (C) (2) and adriamycin (A) (3). Higby et al reported activity of combinations of P + A and C + A in the treatment of a very small group of patients with adenocarcinoma (ACA) or squamous cell carcinoma of the lung (4). Rossof et al reported a response in a previously untreated patient with ACA who was treated with P alone (5). We subsequently observed a 42% response rate to a combination of C, A, and P (CAP-I) in a larger group of patients who had not received previous chemotherapy (6). In an effort to evaluate the contribution of P to the response seen with CAP-I in the treatment of ACA, we performed a randomized study comparing a patient group treated with P as a single agent followed by C-A at the time of progression or the presence of renal toxic effects with a second group of patients treated with CAP-I.

METHODS AND MATERIALS

Forty-one patients with histologically or cytologically proven metastatic ACA of the lung were entered in the study from January 1976 to June 1977. After stratification for Eastern Cooperative Oncology Group performance scores (PS) of 0–1 versus 2–3 and measurable versus evaluable disease, patients were randomized into two treatment groups, the characteristics of which are summarized in table 1. There were no significant differences between the two groups.

Group I (P followed by C-A) received P at a dose of 15 mg/m²/day by iv push on Days 1–5 of a 28-day cycle, as was current practice when the study was initiated. At the time of progression or the presence of unacceptable renal toxic effects at the time of re-

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TABLE 1.—Profile of patients by treatment group

	Treatment group	
	P followed by C-A	CAP-I
No. of patients	22	19
Age in yrs		
Median	60	60
Range	46–77	26–75
Sex		
Men	13	9
Women	9	10
Prior radiation therapy	4	1
PS*		
0–1	15	11
2–3	7	8
Metastasis†		
Lung	5	4
Liver	5	4
Nodes	10	8
Bone	3	3
Central nervous system	6	1
Measurable disease	13	11
Evaluable disease	9	8

* Eastern Cooperative Oncology Group scale.
† Includes multiple sites in some patients.

treatment (serum creatinine ≥ 1.5 mg/100 ml), patients were crossed over to the combination of C-A, ie, C at a dose of 400 mg/m^2 and A at a dose of 40 mg/m^2, both given by iv push on Day 1 of a 28-day cycle. Group II received CAP-I, consisting of C (400 mg/m^2), A (40 mg/m^2), and P (40 mg/m^2), all given by iv push on Day 1 of a 28-day cycle. Upon progression, patients received VP-16-213 at a dose of 140 mg/m^2 iv over 20 minutes on Days 1, 3, and 5 of a 28-day cycle.

Our standard criteria for evaluation of response were used (6). Indicator lesions were either measurable or evaluable. Measurable lesions required clearly defined tumor margins which allowed measurement of the longest perpendicular diameters. Evaluable lesions were visible and assessable but did not allow precise measurement of both perpendicular diameters.

Complete regression (CR) denoted total disappearance of all measurable or evaluable tumor. Partial regression (PR) was defined as a ≥ 50% decrease in the product of the longest perpendicular diameter of the indicator lesions in patients with measurable disease. The term regression applied only to evaluable disease and required a definite decrease in tumor size agreed upon by two investigators. Stable disease implied a < 50% reduction or a < 25% increase in measurable disease and no definite increase or decrease in evaluable indicator lesions. Progression was defined as a ≥ 25% increase in the size of measurable lesions or definite increase in the size of evaluable lesions agreed upon by two investigators or the appearance of new lesions. The duration of response was calculated from the date of determination of response to the date of progression, last followup, or crossover due to drug toxicity. The survival time was calculated from the date of first treatment to the date of death or last followup.

RESULTS

In the P followed by C-A group, two (9%) of 22 patients responded while receiving P. There was one CR and one PR. Both patients had PSs of 0–1. These responses occurred 28 and 85 days after the first treatment and both lasted for 28 days, at which time P was discontinued due to elevated serum creatinine values. Three other patients, who were stable, also developed renal toxic effects which mandated crossover to C-A. One patient withdrew prior to completing his first treatment with P and died of an unknown cause 4 days later. The median time to progression for this group was 63 days.

Seventeen of 21 patients received crossover treatment with C-A. Five patients achieved objective responses with C-A, for a response rate of 29%. Only the five patients with renal toxic reactions to P responded to C-A. No patient whose best response was progression with P responded to C-A. There were four PRs and one CR. Four patients had PSs of 0–1 and one patient had a PS of 2. The median time to response was 57 days (range, 28–233 days). The median duration of response was 276 days (range, 102–533+ days), estimated from Kaplan-Meier plots. The median survival for the five responders was 613 days (range, 258–647 days). One patient was stable after 63 days of treatment with C-A, at which time he withdrew from the study to receive treatment elsewhere. Eleven patients (65%) showed no objective improvement with C-A. The median time to progression was 122 days for all patients receiving P followed by C-A. The median survival for this group was 173 days (range, 90–647 days). The median time to progression and the survival time were estimated from Kaplan-Meier plots. Five patients progressing with C-A received further chemotherapy without benefit: three received 5-fluorouracil (5-FU), one received maytansine, and one received polychemotherapy.

Four of the 21 patients who failed to respond to P did not receive C-A. Two received no further therapy. One patient had no response to VP-16-213 and one achieved a regression with maytansine. The

median survival for these patients was 95 days (range, 28–248 days).

In the 19 patients treated with CAP-I there were four PRs, one CR, and three regressions, for an overall response rate of 42% (eight of 19). Six responders had PSs of 0–1 and two had PSs of 2–3. The median time to response was 72 days (range, 28–155 days). The median duration of response was 95 days (range, 28–173+ days). One patient was stable 137 days after beginning therapy. The median time to progression for the ten nonresponding patients was 57 days (range, 26–150 days). The median time to progression for the entire group was 137 days. The median survival time for patients treated with CAP-I was 205 days (range, 57–438+ days). The median survival time for the eight responders was 197 days (range, 97–438 days).

After failing to respond to CAP-I, 13 of the 19 patients received subsequent therapy. Eight patients received VP-16-213 without objective improvement. Two patients each received maytansine or polychemotherapy. One patient received 5-FU. One of the maytansine-treated patients achieved a regression. There was a response rate of 8% for secondary therapy in this group. Pertinent results are summarized in table 2.

Toxicity

All patients had serum creatinine values < 1.5 mg/100 ml prior to initiating therapy with P or CAP-I. Eight (36%) of 22 patients receiving P and one (5%) of 19 receiving CAP-I developed a serum creatinine value of ≥ 1.5 mg/100 ml (range, 1.5–2.5 mg/100 ml) which mandated discontinuation of P therapy ($P = 0.05$).

For this study, leukopenia was defined as a wbc count of < 4000/mm^3 and thrombocytopenia was defined as a platelet count of < 150,000/mm^3. No patient developed leukopenia while receiving P. Two patients (9%) developed thrombocytopenia (60,000 and 110,000 platelets/mm^3). Following the first course of C-A, eight (47%) of 17 patients developed leukopenia with a median wbc count nadir of 2500/mm^3 (range, 1200–3500/mm^3). One patient developed thrombocytopenia (120,000 platelets/mm^3). Following the first course of CAP-I, 14 (74%) of 19 patients developed leukopenia with a median wbc count nadir of 2700/mm^3 (range, 900–3800/mm^3). One patient developed thrombocytopenia (47,000 platelets/mm^3).

Nausea and/or vomiting of a mild to moderate degree occurred in 11 of 22 patients who received P and in 14 of 19 patients who received CAP-I. There was severe nausea or vomiting in eight of 22 patients receiving P and in four of 19 patients receiving CAP-I.

DISCUSSION

The response rate in P-treated patients was 9%. This level of activity against ACA is comparable to single-agent response rates reported for A, bleomycin, C, hexamethylmelamine, and procarbazine (7).

Trials using C and A or P as initial therapy for ACA have been too limited in size to allow valid

TABLE 2.—Summary of treatment results

	Treatment		
	P	P followed by C-A	CAP-I
No. of patients treated	22	17	19
No. of objective responses (%)	2(9)	5(29)	8(42)*
Median No. of days for—			
Time to response	56	57	72
Duration of response	28	276†	95
Time to progression	63	122	137
Survival			
Entire group	‡	173§	205
Responding patients	‡	613§	197
No. of patients with—			
Subsequent treatment following progression	19	5	13
Response to subsequent treatment	6	0	1

*CAP-I vs P ($P \leq 0.025$).
†P followed by C-A vs CAP-I ($P = 0.05$ Gehan-Wilcoxon).
‡Determined by response to C-A.
§From start of P treatment.

comparisons. The 29% response rate to C-A following P is similar to the 17% response rate to C and A reported by Herman et al (8) and the 23% rate reported by Higby et al (4). There was no significant difference between response rates to P versus C-A or between P followed by C-A versus CAP-I. The number of patients treated is too small to allow correlation of response rates with metastatic sites. In both arms of the study, treatment was continued as long as the patient was stable or had demonstrated a beneficial response. In both groups a median of two treatment cycles was required prior to the observation of improvement.

The 42% response rate for CAP-I is comparable to our earlier results reported for a group of patients with ACA treated with the same regimen (6). There is a significant improvement in the response rate with CAP-I versus P alone ($P \leq 0.025$). The median survival for the CAP-I-treated patients (205 days) in this study is not significantly less than the median survival of the subset of patients with ACA in our first CAP-I treatment study (260 days; $P = 0.39$). Using the same criteria of response as in our first study, the survival of patients responding to CAP-I in the present study, however, is significantly shorter than the survival of ACA responders in our first study (197 days versus 452 days; $P = 0.02$). There is no significant difference in median survival of nonresponders between the CAP-I-treated patients in the present study (220 days) versus the first study (105 days; $P > 0.3$). The groups appear to be well matched by age, PS, and extent of disease; there were more female patients in the current study. The difference in survival of the groups may be due to differences in secondary treatment.

The duration of response was significantly longer for patients responding to C-A after P (median, 276 days) as compared to patients responding to CAP-I in this study (median, 95 days; $P = 0.05$ by Gehan-Wilcoxon method). Although the mechanism responsible for both the reproducibly good response rate with CAP-I and the prolongation of duration of response with P followed by C-A is conjectural, the data suggest that, whether given concurrently or as separate regimens in sequence, there is an interaction between P and C-A which enhances the therapeutic effect of each.

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Phase II Trial of Adriamycin and *cis*-Dichlorodiammineplatinum(II) in Squamous Cell, Ovarian, and Testicular Carcinomas [1]

Brief Reports and Preliminary Communications

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When used alone, adriamycin (ADR) and *cis*-dichlorodiammineplatinum(II) (DDP) have shown activity against testicular, ovarian, and squamous cell carcinomas (1,2). To test the efficacy of this combination against tumors no longer responding to conventional treatment, patients with ovarian, testicular, or squamous cell tumors were treated with the combination of ADR-DDP described below.

METHODS

Eligibility criteria included measurable lesions in patients with histologically confirmed ovarian, nonseminomatous testicular, or squamous cell carcinoma, and an estimated survival of 2 months. Other eligibility requirements included: wbc counts > 4000/mm^3, platelet counts > 150,000/mm^3, BUN < 25 mg/100 ml, serum creatinine < 1.5 mg/100 ml, and creatinine clearance > 75 ml/minute. The drugs were given every 3 weeks.

Complete remission (CR) was defined as the complete disappearance of all pre-existing, measurable lesions with no new lesions appearing. Partial remission (PR) was defined as a 50% reduction of the sum of the products of the largest diameter and its perpendicular for each measurable lesion. Stable disease was defined as a < 50% reduction or a < 25% increase in the measurable lesions. Progression was defined as a ≥ 25% increase in the sum of the products of the diameter of the measurable lesions or the appearance of a new lesion.

Hospitalized patients received 3000 ml of iv fluids on the day preceding and on the day of infusion of the drugs. Outpatients received 1000 ml of half-normal saline over 2 hours immediately prior to infusion of the drugs. ADR (50 mg/m^2) was injected iv over a 2–4-minute period. Immediately afterwards, DDP (50 mg/m^2) was infused at a rate of 1 mg/minute.

RESULTS

Forty-nine patients received ADR-DDP and 43 were evaluable for tumor response. There were four early tumor-related deaths which occurred within 21 days after the first dose of ADR-DDP and one drug-related death due to septicemia during severe leukopenia (300 cells/mm^3). One patient refused further therapy because of severe nausea and vomiting after one course of therapy. The evaluable patients received from one to ten courses of ADR-DDP, with a mean of 4.3 courses per patient.

Response rates and durations of response are shown in table 1. Responses in 16 patients with squamous cell carcinoma of the head and neck included five PRs with three patients having local recurrence, one patient having pulmonary metastases, and one patient having local recurrence and pulmonary metastases. A CR was observed in a previously untreated patient who had laryngeal carcinoma with cervical and pulmonary metastases. Five of these patients had received prior radiotherapy and two had received methotrexate. A 3-month PR was observed in one patient who had an ovarian teratoma in which squamous cell carcinoma was the predominant cell type. There was one PR in a patient with hepatic metastases out of seven patients with squamous cell bronchogenic carcinoma.

Six PRs were observed in 11 patients who had alkylating-agent-resistant ovarian carcinoma, with five responses involving pelvic and abdominal masses and one response involving pulmonary nodules. In six patients with metastatic nonseminomatous testicular cancer there were two PRs and one CR, with all responses involving pulmonary nodules. The three responders had been treated previously with actinomycin D alone rather than with combination chemotherapy.

TOXICITY

Toxic effects are summarized in table 2. In the 24 patients who had nadir counts, the average wbc

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TABLE 1.—Type and duration of response in patients receiving ADR-DDP

Tumor type	Total No. of patients	Response					Duration of response (mos)	
		CR	PR	Stable disease	Progression	% CR + PR	Range	Median
Head and neck carcinoma	16	1	5	4	6	37	1–16*	4.5
Bronchogenic carcinoma	7	0	1	2	4	14	6	6
Miscellaneous squamous cell carcinoma	3	0	1	1	1	33	3	3
Ovarian adenocarcinoma	11	0	6	3	2	54	5–8	6
Nonseminomatous testicular carcinoma	6	1	2	2	1	50	2–8	3

*1 patient with head and neck cancer who achieved a PR died 1 mo later of pneumonia.

TABLE 2.—Toxic effects

Toxic effect	Patients with toxicity		Total No. of evaluable patients
	No.	%	
Nausea and vomiting	49	100	49
Alopecia	49	100	49
Nadir wbc count $< 2000/mm^3$	6	25	24
Nadir platelet count $< 100,000/mm^3$	5	25	24
Hemoglobin decrease > 2 g	18	42	43
Paresthesias	2	5	43

count nadir was $2900/mm^3$ and the average platelet count nadir was $230,000/mm^3$. Nadir counts usually occurred 10–14 days after treatment and returned to levels adequate for further treatment by Day 21. There was one episode of fatal septicemia in a cachectic patient whose wbc count fell to $300/mm^3$. Leukopenia (< 2000 cells/mm^3) and thrombocytopenia ($< 100,000$ cells/mm^3) seemed to be related to the following factors: previous alkylating agent (six patients), previous pelvic radiation (five patients), or poor nutritional status (two patients). There were no episodes of bleeding, and no patient required platelet transfusions. A decrease of > 2 g of hemoglobin, which did not appear to be related to tumor progression, occurred in 18 patients. Anemia, leukopenia, and thrombocytopenia increased in proportion to the number of courses of ADR-DDP.

Serum creatinine rose above normal in two patients with peak levels of 2.0 and 2.5 mg/100 ml. The creatinine levels returned to normal within 5 weeks in one patient and within 4 months in the other. In an attempt to prevent nephrotoxicity all patients received pretreatment hydration. Most patients received 3 liters of iv fluid on the day preceding and on the day of treatment. The two instances of elevated creatinine levels occurred in patients hydrated by this method. No elevations of serum creatinine were seen in 11 patients who received 37 courses of ADR-DDP with 1000 ml of half-normal saline given over 2 hours immediately before the drugs.

Nausea and vomiting occurred in every patient and was severe in two. Every patient experienced alopecia which was reversible when the drugs were stopped. Two patients complained of paresthesias in their hands and feet, but strength and coordination were normal.

DISCUSSION

Previous reports on squamous cell carcinoma of the head and neck have shown a 19% response rate for ADR and a 26% response rate for DDP used at a high dose (3 mg/kg) (3). In the 16 patients receiving ADR-DDP, six responses (37%) were observed, suggesting a virtually additive effect. ADR-DDP produced responses in two patients who had previously failed to respond to methotrexate, indicating a lack of cross-resistance.

There were six PRs among 11 patients whose ovarian carcinoma had failed to respond to treatment with an alkylating agent, indicating a lack of cross-resistance. Response rates for ovarian carcinoma which had failed alkylating-agent therapy are 16% for ADR (1) and 25% for DDP (2). The 53% response rate observed in this study and the 67% response rate for ovarian carcinoma reported by

Bruckner et al (4) suggest a synergistic effect for ADR-DDP. ADR-DDP produced one CR and two PRs in six patients whose nonseminomatous testicular tumors had failed to respond to actinomycin D, while DDP alone produced two CRs and two PRs in seven previously treated patients (5).

In general, leukopenia and thrombocytopenia were mild and were similar to the observations of Vogl et al (6). Normochromic, normocytic anemia may have been due to DDP since Rossof et al (7) have observed anemia with DDP.

Previous clinical trials have shown that DDP is nephrotoxic (1). The relatively mild nephrotoxicity observed in our patients may be due to the pretreatment hydration or to the modest dose of DDP used.

In summary, the combination of ADR-DDP shows promising activity in head and neck carcinoma and in ovarian adenocarcinoma. Nephrotoxicity with this dose and schedule of DDP is mild. Our preliminary results with DDP (50 mg/m^2 every 3 weeks) show that iv administration of 1000 ml of half-normal saline over 2 hours immediately before DDP infusion may be sufficient to prevent nephrotoxicity. Anemia, nausea, and vomiting are considerable. Leukopenia and thrombocytopenia are mild, except in patients who had received abdominal radiation therapy or prolonged therapy with an alkylating agent.

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Adriamycin and Chlorambucil Versus Adriamycin and Thiabendazole in the Treatment of Extensive, Non-Small Cell Carcinoma of the Lung: A Southwest Oncology Group Pilot Study [1,2]

Brief Reports and Preliminary Communications

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In spite of their ability to produce objective response in ≥ 20% of patients, adriamycin-based combinations have been largely ineffective in terms of survival impact on extensive, non-small cell lung cancer. In 1976, the Southwest Oncology Group (SWOG) completed a study (SWOG-7439) which compared two such combinations in patients with squamous cell histology (1). The median survival time (MST) was 16 weeks overall, with no significant difference between the two combinations. Although 18% of all patients entered in the study (total, 247 patients) achieved an objective response, and the responders had significantly longer survival than nonresponders, this was not reflected by any improvement in long-term survival. Possible explanations for this included brevity of responses observed and, most importantly, the toxicity of the regimens employed. Drug-related death occurred in 4% of the patients, and life-threatening toxicity in an additional 9%. This degree of morbidity has been observed in several trials of combination chemotherapy for patients with extensive disease (2–4), and may counterbalance any favorable effects exerted on survival by achieving a response.

Based on these considerations and on the evidence that adriamycin alone produced objective response as frequently as our combinations (5,6), several institutions of the SWOG began a study in 1976 of adriamycin + chlorambucil (AC) versus adriamycin + thiabendazole (AT). Chlorambucil was selected for the following reasons: (*a*) combinations of adriamycin and alkylating agents appear to be synergistic, both in experimental tumor systems (7) and in some human tumors (8,9); (*b*) chlorambucil, given in a high-dose intermittent schedule at a mean dose of 30 mg/m² every 2–3 weeks, had been shown to be biologically effective in the treatment of chronic lymphocytic leukemia (10); and (*c*) at this dose and schedule, chlorambucil appeared to be relatively nonmyelosuppressive. Thiabendazole was chosen as another agent to combine with adriamycin because it had been shown to have immunomodulatory effects on T-cell function similar to levamisole and, in mice, it appeared to be capable of abrogating some of the immunodepressive effects of adriamycin (11). Thus, we hoped that by combining adriamycin with each of two agents which might potentiate its antitumor effects, and which in themselves had relatively little toxicity, the therapeutic index of adriamycin combinations could be improved.

MATERIALS AND METHODS

Patients were eligible for this study (SWOG-7766) in the participating institutions if they had histologically documented, non-small cell carcinoma of the lung which qualified as extensive by the following definition: clinical evidence of disease spread beyond the hemithorax and supraclavicular nodes and/or prior radiation therapy to the chest with local tumor regrowth. They were not eligible for this study if they had received prior chemotherapy. Informed consent was obtained from each patient to participate in a randomized study. Registrations were carried out through the SWOG biostatistical office, and the patients were randomly allocated to treatment with AC or AT after stratification by pretreatment performance score (PS). PS stratifica-

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TABLE 1.—Cell type and PS

	No. of patients receiving—	
	AC	AT
Cell type		
Squamous cell carcinoma	12	17
Adenocarcinoma	9	10
Large cell undifferentiated carcinoma	11	6
PS		
8–10	10	9
5–7	14	17
1–4	8	7

tion, by the modified Karnofsky scale used in our previous study (1), was as follows: PS 8–10 (fully ambulatory); PS 5–7 (partially ambulatory); and PS 1–4 (bedridden). Table 1 shows the patient distribution in each group by histology and PS. There were no automatic exclusions due to other medical problems, except for the presence of uncontrolled heart failure.

In each treatment arm, the dose of adriamycin was 50 mg/m² given iv into a freely running infusion on Day 1 and then every 3 weeks to a total dose of 450 mg/m², at which time it was to be permanently discontinued. In the AC arm, the dose of chlorambucil was 30 mg/m² given orally on Day 1 and then every 3 weeks to disease progression or a period of 2 years in the study. In the AT arm, the dose of thiabendazole was 500 mg on 2 consecutive days of each week (< 2 m² of body surface area) or 1000 mg on 2 consecutive days of each week (≥ 2 m² of body surface area). Thiabendazole was not administered on the same day as adriamycin or within 2 days of an anticipated adriamycin course. The doses of adriamycin and chlorambucil were appropriately modified for each subsequent course, depending on the degree of myelosuppression. Thiabendazole doses were not modified.

Cbc counts were carried out initially at weekly intervals, then every 2–3 weeks if no problems were encountered with myelosuppression. Physical examination, chest roentgenogram, and electrocardiogram were mandatory prior to each course of chemotherapy, as were blood tests of hepatic and renal function; there was no attempt to carry out immunologic monitoring.

The definitions of complete response (CR) and partial response (PR) are the standard definitions employed by the SWOG (1). A $> 50\%$ regression of measurable disease for ≥ 4 weeks was considered a PR.

RESULTS

A total of 70 patients were entered in the study. At this time, it is too early to evaluate five of these patients. The remaining 65 patients are the subject of this paper. Response was noted in three of 32 patients (9%) who were randomized to AC and in five of 33 patients (15%) who were randomized to AT. Improvement, but less than that required for PR, was noted in three additional patients receiving AC and in four patients receiving AT (PR + improved, 19% versus 27% respectively). None of these differences between AC and AT are statistically significant.

The MST was 16.5 weeks for patients with squamous cell histologies and was essentially the same (19 weeks) for adenocarcinoma and large cell undifferentiated carcinoma. The MST for AC was 20 weeks versus 16 weeks for AT (not significantly different). As in our previous studies, patients who were fully ambulatory before treatment (PS 8–10) had superior survival (20 weeks) compared to those who were entered with lesser degrees of PS (PS 1–7; 16 weeks). This was significant at a level of $P = 0.057$. No significant differences in survival were noted between the two treatment arms within the PS categories. There were also no significant differences in survival when comparisons were made by histologic subtype.

Since there were no survival differences related to cell type, the survival curve for all extensive non-small cell patients entered in this study was compared to that from our previous, group-wide study (bleomycin, adriamycin, CCNU, vincristine, and mechlorethamine [BACON] versus mechlorethamine, adriamycin, and CCNU [NAC]) which was open only to extensive-disease patients with squamous cell histology. This comparison is shown in figure 1. The two curves are superimposable.

The toxicity from this program was primarily related to myelosuppression. Among the patients receiving AT, there were six cases of life-threatening toxicity, five of which were related to granulocytopenia and infection, and three deaths. The median nadir granulocyte count was 1100/mm³ for AC and 1300/mm³ for AT, with no difference in the frequency of granulocyte depression < 500/mm³ (AC, 35% versus AT, 15%). Most patients' platelet counts did not drop to $< 50{,}000$/mm³ on either regimen, although there was a suggestion of greater severity on the AC regimen when this complication did occur (18% of total AC courses associated with platelet nadir $< 50{,}000$/mm³ versus 0 for AT).

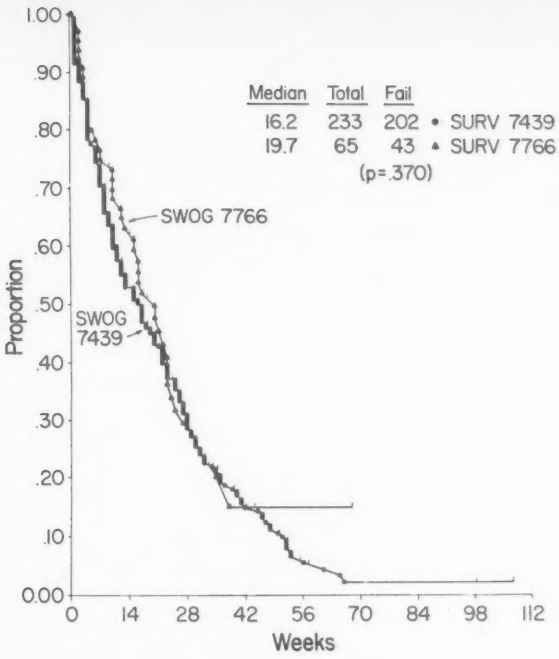

FIGURE 1.—Survival comparison between SWOG-7439 and SWOG-7766 (present study).

DISCUSSION

The results of this study show no advantage for either of the two simple, adriamycin-based combination chemotherapy programs compared to each other or to historical control results for supportive care or adriamycin alone (12,13). They were associated with only slightly less life-threatening toxicity than previous BACON or NAC regimens (9% versus 13%), and the frequency of drug-related deaths was the same (4%). It is of interest that AC produced only slightly more leukopenia than AT, and it is possible that the dose of chlorambucil which we selected was suboptimal. At least one other combination of adriamycin and an alkylating agent (cyclophosphamide) has been reported to show significant survival benefit among extensive-disease patients with squamous cell histology (14). We do not recommend further exploration in patients with extensive disease with either regimen used in this study.

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Cytologic Evidence That Taxol, an Antineoplastic Agent From *Taxus brevifolia*, Acts as a Mitotic Spindle Poison [1,2]

Brief Reports and Preliminary Communications

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Taxol is a plant product with a unique and complex structure which was isolated from *Taxus brevifolia* (1). Taxol is being considered as a candidate for development to clinical trial in cancer based on its activity against P388 leukemia and B16 melanoma. We observed that a subline of P388 leukemia resistant to adriamycin (2) was cross-resistant to taxol (3). This adriamycin-resistant line is cross-resistant to a spectrum of cytotoxic drugs which act by one of the following mechanisms: (*a*) DNA intercalation, (*b*) interference with mitotic spindle function, or (*c*) inhibition of protein biosynthesis (3). Because of this observation, we initiated a number of experiments to determine whether taxol acts by one of these three mechanisms.

MATERIALS AND METHODS

Taxol was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. It was dissolved in *N,N*-dimethylacetamide, followed by the addition of an equal volume of Emulphor 620 (GAF Corp, New York, NY) and subsequently by eight volumes of 0.85% NaCl solution, for injection into tumor-bearing mice. The resultant solution of taxol (10 mg/ml) was injected immediately sc in a volume of 0.01 ml/g of body weight (100 mg/kg). Taxol, which is virtually insoluble in aqueous medium, remained in solution at room temperature in this formulation for 10 minutes.

P388 leukemia was inoculated ip at 10^6 viable cells/mouse into a large group of (C57BL/6 × DBA/2)F_1 [B6D2F_1] male mice. The mice were treated with taxol 7 days after tumor inoculation when leukemic ascites was evident. A small volume of ascites fluid was collected from groups of three mice at 2-hour intervals up through 24 hours after drug administration and at 28, 32, 36, 48, 72, and 96 hours. Smears were made with ascites fluid from each animal and stained with Giemsa stain. Five hundred nucleated, intact cells were counted on each slide, and the mitotic index (MI) was quantitated. The slides were counted blindly, that is, the person counting the slides was not informed of which treatment group or time-point the slides represented. Smears were also obtained from vehicle-treated animals at 0, 10, 24, 48, and 72 hours.

To determine the effect of taxol on cell size, 50 interphase cells at each time-point were measured microscopically using an ocular micrometer and the average cell size was determined.

RESULTS AND DISCUSSION

It was readily apparent from the morphology of P388 cells taken from taxol-treated mice that the drug produces mitotic abnormalities (fig 1). Disrupted and abnormal mitotic figures were seen at all time-points following administration of 100 mg/kg of taxol, a dose which is well-tolerated by mice. The mitotic figures are similar to those obtained following administration of known mitotic spindle poisons such as vinca alkaloids (4,5) or maytansine (6).

The stathmokinetic effect of taxol on P388 leukemia cells in vivo is shown in figure 2. In control mice the MI of P388 leukemia varied between 1% and 2%. After taxol treatment the MI progressively increased to 8% at 12 hours. The MI returned to control levels 14 hours after drug administration. There was a great deal of variability associated with the MI 12 hours following taxol administration; the values for the three animals were 2.2%, 6.8%, and 15.4%, indicating that in one animal the stathmokinetic effect of the drug was over by 12 hours. Taxol is much less efficient than other mitotic spindle poisons in producing mitotic arrest; in a similar in vivo experiment maytansine arrested up to 60% of P388 cells in mitosis (7), and vinca alkaloids have been shown to increase the MI of leukemia cells to about 30% (8,9).

[1]Received Mar 10, 1978; accepted May 24, 1978.

[2]Taxol: NSC-125973; 5β,20-epoxy-1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one, 4,10-diacetate, 2-benzoate, 13-(α-phenyl hippurate).

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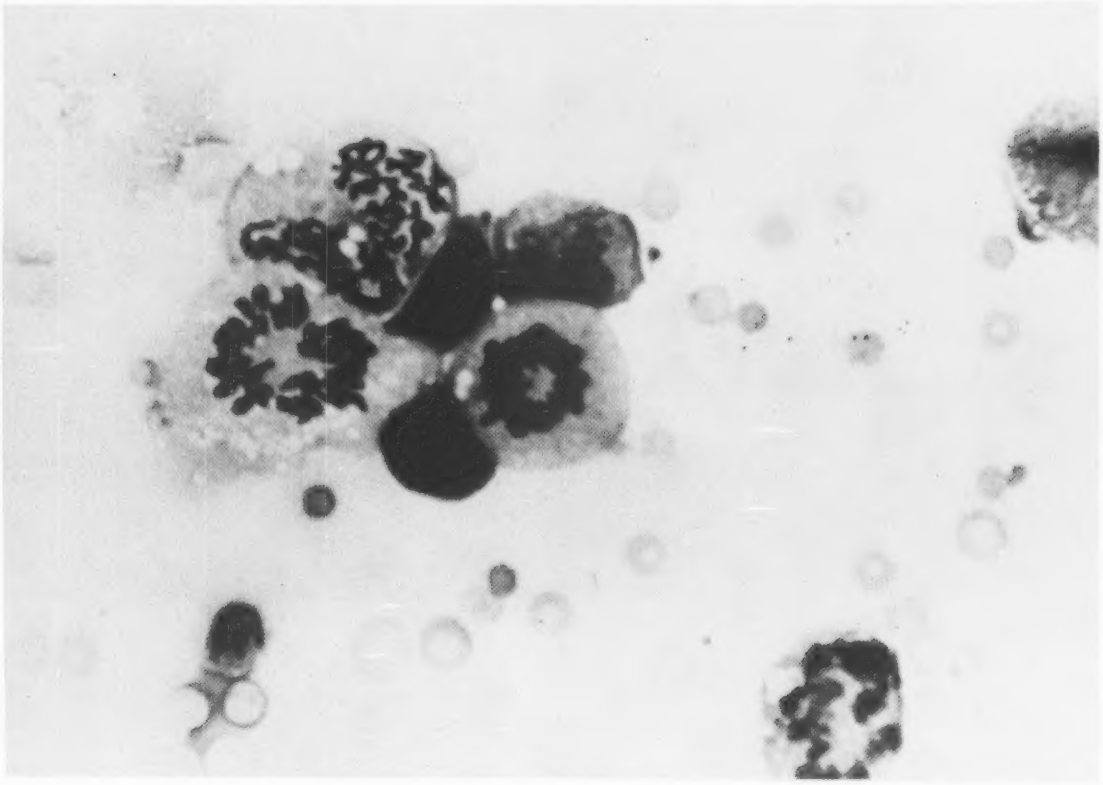

FIGURE 1.—Ascites cells from a mouse bearing P388 leukemia 12 hrs after administration of taxol, 100 mg/kg sc (Giemsa stain, × 500).

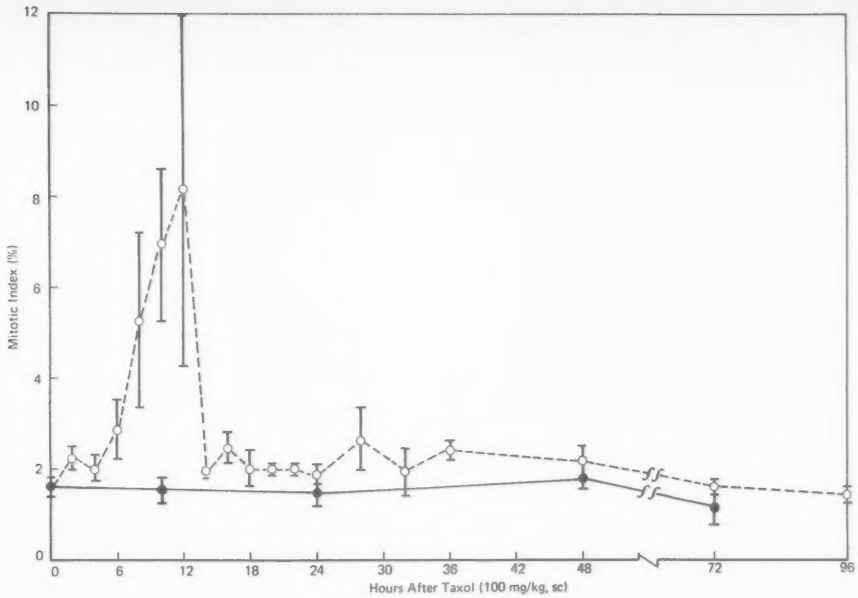

FIGURE 2.—MI of P388 leukemia cells taken from tumor-bearing mice at various times after administration of taxol (○-----○). Each point is the average of 3 mice and the error bars = SEM. Value at 10 hrs is significantly different ($P < 0.05$) from vehicle-treated controls (●——●).

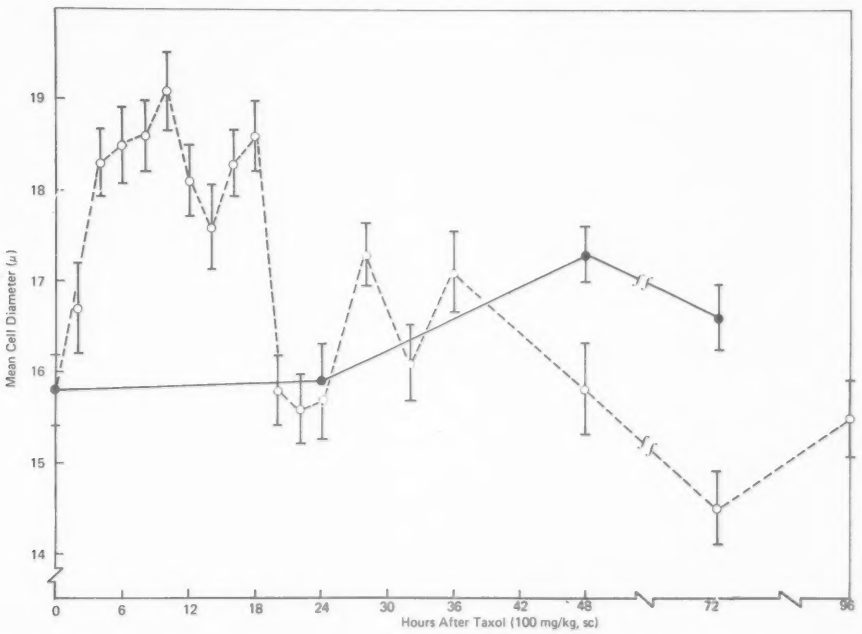

FIGURE 3.—Mean diameter of interphase P388 leukemia cells taken from tumor-bearing mice at various times after administration of taxol (○----○). Each point is the average of 50 cells and the error bars = SEM. Values at 4–18 and 72 hrs are significantly different ($P < 0.05$) from vehicle-treated controls (●——●).

Taxol also produced a time-dependent increase in the size of interphase P388 leukemia cells (fig 3). Increased cell size was evident from 4 to 18 hours after administration of taxol. The relevance of this observation to the mechanism of action of taxol is not known. There was no evidence of an increase in the number of interphase cells with multiple and/or bizarre nuclei as was observed following maytansine administration (7).

These results indicate that taxol has antimitotic activity similar to that observed with vinca alkaloids and maytansine. It has less stathmokinetic activity in vivo than the other mitotic spindle poisons. Investigations on the binding of taxol to tubulin may provide more insight into its antitumor activity.

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Phase I–II Study of Intermittent Bolus Administration of DTIC and Actinomycin D in Metastatic Malignant Melanoma [1,2]

Brief Reports and Preliminary Communications

Michael K. Samson,* Laurence H. Baker, Robert W. Talley, Roberto J. Fraile, and Bruce McDonald [3]

SUMMARY

Twenty-six patients with disseminated malignant melanoma were treated with intermittent bolus DTIC and actinomycin D in an escalating dose schedule, starting at 650 and 1 mg/m² respectively. Courses were repeated at 3–4-week intervals. Twenty-four patients were evaluable for toxicity and 22 were evaluable for response. Two patients (9%) had a complete remission lasting 7+ and 14 months, and three patients (14%) had a partial remission lasting 2+, 5+, and 14+ months. Nausea and vomiting, lasting 24 hours, was observed in 88% of patients, while diarrhea was noted in 17%. Stomatitis and alopecia were less frequently observed. All responses occurred at nonmyelosuppressive doses and in patients with visceral-predominant metastases. This schedule offers the patient the convenience of single-day treatment and less prolonged gastrointestinal intolerance. Further evaluation of this drug combination and schedule would appear to be indicated.

[Cancer Treat Rep 62:1223–1225, 1978]

DTIC is a drug with an unknown mechanism of action, although it is structurally similar to 4-amino-5-imidazole carboxamide, a purine precursor. In several clinical studies, it appears to be the single most active drug in the treatment of disseminated malignant melanoma (1–3). In most studies, DTIC was administered as a single iv injection for 5 consecutive days with subsequent courses given at 21–28-day intervals. Maximum wbc and platelet count depressions occurred at 21–25 days after initiation of therapy and recovery occurred 3–5 days later. This schedule has often met with poor patient compliance because of protracted gastrointestinal intolerance. Cowan and Bergsagel (4) reported that the intermittent single-dose administration of DTIC could be given safely without sacrificing antitumor effect, confirming animal experimental data which suggested that the drug was not schedule dependent. Significant leukopenia and thrombocytopenia were observed at a dose of 850 mg/m², with nadirs reached at 21 and 16 days respectively and recovery noted at 29 and 21 days. Their data were substantiated by Pritchard et al (5) in a more recent study.

Benjamin et al (6) have reported the use of actinomycin D in an intermittent treatment schedule in disseminated melanoma. The rationale for such a schedule was based on animal and clinical data that indicated a long plasma half-life (7,8). They concluded that a dose of 2 mg/m² iv every 3–4 weeks resulted in tolerable hematologic and nonhematologic toxicity.

The combination of DTIC and actinomycin D appeared to be synergistic in the L1210 leukemia system.[4] A recent clinical trial of DTIC and actinomycin D in Kaposi's sarcoma resulted in a high response rate and suggested therapeutic synergy (9). Thus, it appeared reasonable to combine these two drugs, administered on a single-dose intermittent basis, in untreated patients with disseminated malignant melanoma.

MATERIALS AND METHODS

Twenty-six patients (17 men and nine women) with disseminated malignant melanoma were entered in this study. None had received prior chemotherapy. The only therapy given to all patients prior to entry was surgical. Patients were evaluated before therapy with regard to tumor measurements as well as enzymatic and hematologic parameters. Twenty-four patients were evaluable for toxicity and 22 were evaluable for response. One patient

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[2] Supported by contract N01-CM-67105 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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[4] Von Hoff DD, and Abraham D. Personal communication.

died of tumor 10 days after the initiation of therapy and was considered unevaluable because of early death. Three patients were lost to followup. All but seven patients had extensive extranodal disease, including brain, lung, and/or liver metastases. The mean age of the patients was 57 years (range, 35–85). The mean Karnofsky performance status was 85 (range, 50–90).

DTIC was administered iv over 15–20 minutes followed by actinomycin D on Day 1, and repeated at 3–4-week intervals. Six different dose escalation schedules were evaluated for toxicity and response and were as follows: schedule 1, DTIC (650 mg/m^2) and actinomycin D (1 mg/m^2); schedule 2, DTIC (750 mg/m^2) and actinomycin D (1 mg/m^2); schedule 3, DTIC (750 mg/m^2) and actinomycin D (1.5 mg/m^2); schedule 4, DTIC (800 mg/m^2) and actinomycin D (1.7 mg/m^2); schedule 5, DTIC (850 mg/m^2) and actinomycin D (1.7 mg/m^2); and schedule 6, DTIC (950 mg/m^2) and actinomycin D (2 mg/m^2). In the absence of toxicity, dose escalation to the next highest treatment schedule was encouraged. If a response was observed, patients were continued on the same schedule until relapse was documented. A patient was considered to have received an adequate trial if two or more courses of therapy were given or if increasing disease (> 25%) was noted after one course of therapy.

Complete remission was defined as the disappearance of all clinical evidence of active tumor for a minimum of 4 weeks together with the disappearance of all tumor-related symptoms. Partial remission was defined as a ≥ 50% decrease in the sum of the products of the largest perpendicular diameters of all measured lesions lasting ≥ 4 weeks in the absence of a significant increase in any one lesion or the appearance of new lesions. Disease progression was defined as an unequivocal increase of any measured lesion or the appearance of new lesions.

RESULTS

Response

Of the 22 evaluable patients, two (9%) had a complete remission lasting 7+ months (schedule 3) and 14 months (schedule 1) respectively, and three (14%) had a partial remission lasting 2+ months (schedule 2), 5+ months (schedule 1), and 14+ months (schedule 1) respectively, for an overall response rate of 23%. Table 1 correlates response with metastatic site. Significantly, all responders had visceral-dominant metastatic disease. No responses were observed on schedules 4, 5, and 6.

TABLE 1.—Response by site of metastases

Metastatic site*	No. of patients with—		
	Complete remission	Partial remission	Increasing disease
Lung	2	—	8
Liver	—	2	4
Brain	—	—	2
Skin	—	—	4
Lymph nodes	1	2	10
Bone	—	—	2

* Most patients had multiple metastatic sites.

Toxicity

Table 2 lists the hematologic toxicity (leukopenia and thrombocytopenia) and the number of patients treated by schedule. No significant myelotoxicity was observed until schedule 6 was reached. At that level, three of five patients had significant leukopenia (< 3000 cells/mm^3) and thrombocytopenia (< 100,000 cells/mm^3). The mean days to nadir was 15 for the wbc count and 10 for the platelet count. Recovery for both elements was noted by Day 16. There was no delay in administering second or subsequent courses of therapy due to prolonged bone marrow toxicity, nor was there any evidence of cumulative toxicity. No patient required hospitalization for infection or hemorrhagic complications of myelosuppression. There were no drug-related deaths although one patient developed life-threatening leukopenia and thrombocytopenia (schedule 6). Nonhematologic toxicity could be evaluated in 24 patients. Severe nausea and vomiting, poorly controlled by antiemetics, was observed in 88% of the patients. Typically, these symptoms would persist for approximately 24 hours and then spontaneously resolve. Diarrhea was noted in 17% of the patients and would persist for up to 3–4 days. Stomatitis and alopecia were less frequently observed.

DISCUSSION

This study was designed to take advantage of the lack of schedule dependency that has been shown for DTIC and actinomycin D. Furthermore, animal data utilizing a single-dose intermittent schedule of actinomycin D suggest that a higher therapeutic index may be achieved with such a schedule, when compared to the standard 5-day loading-dose schedule (10).

There are several significant features of the clinical study. All of the responding patients had visceral-dominant metastatic patterns which have

TABLE 2.—Hematologic toxicity

Schedule	Dose (mg/m^2) of DTIC/ actinomycin D	No. of patients treated	Median wbc nadir ($\times 10^3/mm^3$)	Range	No. of patients with wbc count $< 3000/mm^3$	Median platelet nadir ($\times 10^3/mm^3$)	Range	No. of patients with platelet count $< 100{,}000/mm^3$
1	650/1	3	5.4	4.2–6.5	0	164	160–338	0
2	750/1	4	4.0	3.4–9.7	0	130	93–157	1
3	750/1.5	8	5.1	0.8–8.4	1	170	69–209	1
4	800/1.7	5	5.6	2.1–6.6	1	180	94–315	2
5	850/1.7	3	7.1	3.3–7.2	0	187	100–213	0
6	950/2	5	2.7	0.5–5.0	3	79	15–320	3

been, in past studies, more resistant to chemotherapy. Additionally, the advantages of a convenient 1-day treatment schedule, of less prolonged nausea and vomiting, and of responses occurring without significant myelosuppression are obvious. Recently, Gutterman et al (11) have shown that bolus actinomycin D, when combined with the standard 5-day schedule of DTIC and iv *Corynebacterium parvum* in patients with disseminated malignant melanoma, appeared to be very active. They noted responses in six of 13 patients, with tumor confined to either liver or lung. This suggests that this two-drug combination, DTIC and actinomycin D, may be particularly active in situations where visceral metastases may predominate.

The Southwest Oncology Group is currently exploring intermittent bolus administration of DTIC and actinomycin D compared to BCNU, hydroxyurea, and DTIC (BHD) and BHD + levamisole in disseminated malignant melanoma, to determine if our observations can be confirmed in a considerably larger treatment population. Based on the lack of significant myelosuppression coupled with an antitumor effect, our recommendation for dose and schedule is 750 mg/m^2 of DTIC and 1.7 mg/m^2 of actinomycin D repeated at 3–4-week intervals.

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Screening for New Antitumor Antibiotics of the Bleomycin Group[1]

Brief Reports and Preliminary Communications

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An antitumor antibiotic, bleomycin, is at present widely used clinically for the treatment of some forms of malignant tumors (1). Bleomycin is produced by a culture of actinomycete, *Streptomyces verticillus*, which is isolated from Japanese soil (2). Recently, a rare culture of actinomycete isolated from Indian soil produced the antitumor antibiotic, tallysomycin, related to bleomycin (3). The culture producing tallysomycin was described as *Streptoalloteichus*, a new genus of the family Actinoplanaceae. Experimental studies on animals have shown that tallysomycin has a therapeutic advantage over bleomycin in some malignant tumors (4). These observations suggest that further screening for new antitumor antibiotics of the bleomycin group would be of considerable interest.

The development of new tests designed for effective early recognition of antibiotics of the bleomycin group among various natural compounds synthesized by micro-organisms is of great importance for further screening. Recently, Bradner (5) noted unusual sensitivity of *Penicillium avellaneum* to bleomycin-type antibiotics. This test organism is also sensitive to antifungals (amphotericin and nystatin), but completely resistant to antibacterial antibiotics. We confirmed this observation working with a culture of *P. avellaneum* 444 from our laboratory. The growth of this culture on nutrient agar in Petri dishes was inhibited by bleomycin in a concentration of 0.2 μg/ml.

To make this test more sensitive for recognition of antibiotics of the bleomycin group, we cultivated *P. avellaneum* 444 on nutrient agar in Petri dishes, as well as in nutrient broth, containing increasing concentrations of bleomycin. A new variant of *P. avellaneum* which was 20 times more resistant to bleomycin than the initial culture was developed in this way. It was inhibited by bleomycin at a concentration of 4 μg/ml.

TABLE 1.—Effect of different antibiotics of the bleomycin group upon *P. avellaneum* 444 and its variant resistant to bleomycin

Antibiotic	Minimal inhibitory concentrations (μg/ml)	
	P. avellaneum 444	*P. avellaneum* resistant to bleomycin
Bleomycin complex	0.2	4.0
Bleomycin A_5	0.2	4.0
Bleomycin B_2	0.2	4.0
Bleomycin A_2	2.0	20.0
Tallysomycin	0.5	5.0

The effect of various antibiotics of the bleomycin group upon the parent culture of *P. avellaneum* 444 and its bleomycin-resistant variant is shown in table 1. These results indicate that the variant of *P. avellaneum* developed by us is more resistant not only to various bleomycins, but also to tallysomycin.

Additionally, we studied the inhibitory effect of cultures of micro-organisms producing different antitumor antibiotics upon the growth of *P. avellaneum* 444 and of its resistant variant. The producing cultures were grown on nutrient agar in Petri dishes at 28°C, and after 5 days were streaked with suspensions of test organisms. Table 2 shows that only cultures producing bleomycin inhibited the growth of *P. avellaneum* 444 and to a much smaller extent its variant resistant to bleomycin. Cultures of micro-organisms producing anthracyclines (daunorubicin and carminomycin), actinomycin D, streptonigrin, and other antitumor antibiotics belonging to different groups of chemical compounds did not in any way inhibit cultures of *P. avellaneum*. These cultures are also resistant to the action of antibacterial antibiotics. Antifungal antibiotics inhibit both parent and bleomycin-resistant cultures of *P. avellaneum* to the same extent.

These observations indicate that *P. avellaneum* 444 and its variant resistant to bleomycin are very convenient and specific tests for detection of cultures of micro-organisms producing new antitumor antibiotics of the bleomycin group. This test is used at present in the screening program of the Institute of New Antibiotics.

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TABLE 2.—Effect of cultures of micro-organisms producing different antitumor antibiotics upon growth of *P. avellaneum* 444 and its variant resistant to bleomycin

Antibiotic	Producing culture	Zones of inhibition of growth (mm)	
		P. avellaneum 444	*P. avellaneum* resistant to bleomycin
Bleomycin	*Streptomyces verticillus*	17	7
Bleomycin A_5	*Streptomyces* sp. nov.	18	10
Daunorubicin	*Streptomyces coeruleorubidus* var. *rubomycini*	0	0
Carminomycin	*Actinomadura carminata*	0	0
Actinomycin D	*Streptomyces galbus*	0	0
Streptonigrin	*Streptomyces albus*	0	0
Echinomycin	*Streptomyces echinatus*	0	0
Sibiromycin	*Streptosporangium sibiricum*	0	0
Nocamycin	*Nocardiopsis syringae*	0	0
Virenomycin	*Streptomyces virens*	0	0

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High-Dose 6-Mercaptopurine in Advanced Refractory Cancer [1]

Brief Reports and Preliminary Communications

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6-Mercaptopurine (6-MP) is an active agent in the treatment of acute leukemia in both children and adults (1–3). Activity against solid tumors has been less well studied because daily oral as well as intermittent iv administration schedules of 6-MP were found in the past to be generally ineffective and toxic (4). Advances in supportive care have, however, allowed for more aggressive chemotherapy. Recently, intensive or high-dose (HD) intermittent iv therapy with drugs such as cyclophosphamide and methotrexate has shown activity in several tumors such as small cell bronchogenic carcinoma and osteogenic sarcoma (5,6). There are few studies that have used 6-MP at high doses (eg, 500–1000 mg/m^2/day × 5 days) other than those that have been used to treat acute leukemia. Therefore, this study was undertaken to evaluate the effectiveness and toxicity of HD 6-MP in patients with advanced, refractory cancer in an effort to determine whether a dose and schedule of the drug similar to that used in acute leukemia might result in greater therapeutic activity against solid tumors compared to conventional 6-MP treatment.

MATERIALS AND METHODS

Patient Selection

Patients with histologically documented malignant disease which was measurable and for which no other effective therapy was available were considered eligible for HD 6-MP. In addition, patients were required to have a projected life expectancy of ≥ 2 months, a wbc count > 4000/mm^3, a platelet count > 100,000/mm^3, a bilirubin < 1.2 mg/100 ml, and a serum creatinine < 1.5 mg/100 ml. Hyperuricemia and hypercalcemia, if present, were appropriately corrected prior to treatment.

History and physical examination, blood and differential counts, urinalysis, liver and renal function tests, and appropriate roentgenograms were performed prior to initiation of therapy. Specific studies such as bone marrow aspirate or biopsy, lymphangiogram, intravenous pyelogram, upper or lower gastrointestinal contrast studies, liver, brain, or bone scan, and metastatic bone survey were performed when indicated in order to enumerate all tumor lesions.

Drug Administration

6-MP was given by rapid iv infusion at a dose of 1000 mg/m^2/day for the first 5 days of each 21-day course. The remaining 16 days were allowed for recovery of toxicities. Courses were repeated every 21 days when counts and other toxicities permitted. Allopurinol was not administered.

Response Evaluation

Complete remission (CR) was defined as the complete disappearance of all signs and symptoms of disease including the disappearance of all tumor lesions. Partial remission (PR) was defined as a ≥ 50% regression (in the sum of the products of the perpendicular diameters) of all measurable disease compared with pretreatment measurements lasting > 28 days. Progressive disease was defined as the appearance of new lesions persisting for 2 weeks, an increase of ≥ 50% in the sum of the products of the perpendicular diameters of any of the tumor lesions, or the appearance or progression of abnormal clinical or laboratory parameters related to active tumor.

Patient Characteristics

Twenty patients were treated with HD 6-MP (table 1). Three patients were considered unevaluable for tumor response. One of these three patients died of bacterial sepsis after receiving only the first day of the 5-day course of HD 6-MP and therefore response could not be evaluated. The second patient died from an infection on Day 10 of his first course

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TABLE 1.—Patient characteristics and results of HD 6-MP therapy

Patient No.	Age/ sex/ race*	Diagnosis (primary site)	Metastases	PS	Prior therapy†	Dose (mg/day) × No. of days given	Response
1	16/F/W	Acute myeloblastic leukemia	—	10	Dm,Vc,Ca,Pd,Vb,Az	1500 × 1	None (died next day after being infected)
2	22/M/W	Chronic myelocytic leukemia	—	70	Bu,Vc,Pd,H,Pz	1700 × 5 1700 × 5	No change Progression
3	48/F/B	Adenocarcinoma, unknown primary	Liver, thyroid, bone, brain	20	Ad,RT	1650 × 5	Progression
4	56/M/W	Adamantinoma, unknown primary	Lungs, bone	90	Ad,Cp,Vc,Bl,Mt	1800 × 3	None (refused further therapy)
5	58/M/B	Adenocarcinoma, colon	Skin, epigastric mass	30	F,Cc,Dg	1800 × 5	Progression
6	60/F/W	Melanoma, skin	Skin, lung, brain	30	Dt,Me,Mc	1200 × 5	Progression
7	37/M/W	Melanoma, skin	Nodes, skin	40	Dt,Me	1900 × 5	Progression
8	54/F/B	Infiltrating ductal, breast	Nodes, liver	20	Cp,Vc,Mt,F,Pd,Ad,Vp	1700 × 5	Progression
9	45/F/B	Adenocarcinoma, breast	Nodes, lungs, bone	30	Cp,Vc,Ad,F,Pd	1500 × 5	Progression
10	39/F/B	Infiltrating ductal, breast	Lung, bone, brain	20	Cp,F,Rt	1750 × 5	Progression
11	40/F/W	Infiltrating ductal, breast	Nodes	30	Cp,Vc,Mt,F,Pd,Ad,Vp, RT	2000 × 5 1390 × 5	No change Progression
12	61/F/W	Adenocarcinoma, breast	Lung, bone, brain	50	Cp,Vc,Ad,F,Pd,Rt	1500 × 5 1700 × 5	No change Progression
13	72/M/B	Undifferentiated small cell, lung	Liver, bone	20	Cp,Ad,Vp,Me	1800 × 5	None (died on Day 10 after being infected)
14	57/M/W	Adenocarcinoma, lung	Nodes, liver, bone	20	L,RT	1700 × 5	Progression
15	65/M/W	Undifferentiated large cell, lung	Nodes, bone	30	L,RT	1770 × 5	Progression
16	52/M/W	Squamous cell carcinoma, lung	Lung, bone	30	L,Ad,RT	1800 × 5	Progression
17	69/F/W	Squamous cell carcinoma, lung	Nodes, lung	80	L,Ad,RT	1450 × 5 1450 × 5	No change Progression
18	51/F/B	Adenocarcinoma, lung	Nodes, bone	60	Ad,RT	1450 × 5 1450 × 5	No change Progression
19	61/M/W	Adenocarcinoma, lung	Nodes, chest wall	40	L,Ad,RT	1750 × 5 1600 × 5 1600 × 5	No change No change Progression
20	61/M/W	Undifferentiated large cell, lung	Nodes, liver, brain	80	L,Cp,RT	1830 × 5 1830 × 5 1850 × 5 1850 × 5 1850 × 5	No change No change No change No change Progression

*W = white; B = black.

†Ad = adriamycin; Az = 5-azacytidine; Bl = bleomycin; Bu = busulfan; Ca = cytosine arabinoside; Cc = CCNU; Cp = cyclophosphamide; Dg = dianhydrogalactitol; Dm = daunorubicin; Dt = DTIC; F = 5-fluorouracil; H = hydroxyurea; L = levamisole; Mc = methyl-CCNU; Me = MER; Mt = methotrexate; Pd = prednisone; Pz = piperazinedione; RT = radiotherapy; Vb = vinblastine; Vc = vincristine; Vp = VP-16-213.

of HD 6-MP before any response could be definitely attributed to the chemotherapy. The infectious death was due to leukopenia from HD 6-MP. The third patient refused completion of the first course of HD 6-MP because he was unable to tolerate the severe nausea and vomiting.

The mean age of the 17 evaluable patients was 52 years (range, 22–69). All patients had metastatic disease. The mean Karnofsky performance status (PS) for the 17 evaluable patients was 40 (range, 20–80) and all had had prior treatment. Fifteen patients had received prior chemotherapy and two had received only prior immunotherapy and radiotherapy. Eleven of the 15 chemotherapy patients had also received radiotherapy and six had received immunotherapy.

RESULTS

Response

Seventeen patients received at least one full course of HD 6-MP and were considered evaluable (table 1). There were no CRs or PRs. Ten patients had progressive disease following one course of HD 6-MP and a second course was not attempted. Five patients had no change after the first course and then developed progressive disease after the second course of HD 6-MP. One patient had no change after two courses and another patient had no change after four courses. Both of these patients developed progressive disease after the third and fifth courses respectively.

Toxicity

The toxic effects from HD 6-MP were primarily hematologic and gastrointestinal (table 2). Leukopenia (< 3000 cells/mm^3) and thrombocytopenia (< 100,000 cells/mm^3) were experienced by all patients. Severe leukopenia (< 1000 cells/mm^3) was seen in five patients and severe thrombocytopenia (< 20,000 cells/mm^3) was seen in one patient which lasted 10 days but did not require platelet transfusions. There were three drug-related infections and no drug-related hemorrhages among the 20 patients. There was one drug-related death. This infectious death was due to HD 6-MP-induced leukopenia. Nausea and vomiting occurred in all patients. The vomiting could not be controlled with antiemetics in eight patients. Diarrhea occurred in six patients and necessitated parenteral hydration in three. Mucositis/stomatitis was observed in eight patients and limitation of oral intake was seen in four.

TABLE 2.—HD 6-MP toxicity (20 patients)

Toxic effect	Mean (range)
Hematologic*	
Leukopenia	
Nadir† (cells/mm^3)	1900 (100–4200)
Day of nadir (wbc count < 3000/mm^3)	16 (11–23)
Day of recovery	24 (17–34)
Thrombocytopenia	
Nadir† (cells/mm^3)	75,000 (13,000–200,000)
Day of nadir (platelet count < 100,000/mm^3)	15 (12–21)
Day of recovery	23 (17–30)
Gastrointestinal	*No. of patients*
Nausea	20
Vomiting	20
Diarrhea	6
Mucositis/stomatitis	8

*Tabulated for all courses of therapy given.

†Day of nadir and recovery calculated from 1st day of each 5-day course of therapy.

DISCUSSION

The dose and schedule of 6-MP used in this study do not appear effective for the treatment of advanced, refractory cancer, in particular breast (five patients) and lung (eight patients) cancer. HD 6-MP produced significant hematologic and severe gastrointestinal toxicity. Leukopenia was associated with infection, and there was one drug-related death.

Although increasing the dose of drugs such as cyclophosphamide and methotrexate to increase their therapeutic activity has been successful, increasing the dose of 6-MP was not successful because of increased toxicity without tumor responses in this group of patients. Further studies with 6-MP at this dose and schedule are not warranted.

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Vinblastine and 6-Thioguanine in the Treatment of Advanced Breast Cancer [1,2]

Brief Reports and Preliminary Communications

Hyman B. Muss,* Douglas R. White, Frederick Richards II, M. Robert Cooper, John J. Stuart, and Charles L. Spurr [3,4]

The treatment of the patient with advanced breast cancer whose disease has become refractory to first-line chemotherapy and hormonal therapy represents a frequently encountered and difficult management problem. Responses to secondary chemotherapy regimens are few, usually brief, and of minimal clinical benefit. The present study was undertaken to determine whether the sequential administration of vinblastine followed by 6-thioguanine would be of benefit in patients refractory to a five-drug program which included adriamycin.

Vinblastine, when used as a single agent, has had a response rate of 20% (19 responses among 95 patients) (1). The method of administration has usually been by weekly iv administration. In 55 patients reported in four studies, there were 19 responders (35%) while in five other studies involving 40 patients no responses were noted. These data indicate that the role of vinblastine in the treatment of advanced breast cancer has not been completely defined.

Experience with purine antagonists in patients with breast cancer has been limited. Regelson et al (2) treated four patients without any response but Moore et al (3) reported that six of 44 patients (14%) with advanced breast cancer responded to 6-mercaptopurine (6-MP). Their patients received a dose of 1 mg/kg divided into two daily doses. Esterhay et al (4) have recently treated five patients with high-dose iv 6-MP with two patients showing brief disease stabilization. 6-Thioguanine was selected for our study since it has a wider spectrum of activity in vitro than 6-MP. 6-Thioguanine, unlike 6-MP, may achieve its antitumor effect by incorporation into DNA (5).

The sequential use of vinblastine followed by 6-thioguanine was undertaken with the hope that vinblastine might synchronize as well as destroy dividing cells in tumor tissue and thereby make them more susceptible to destruction by 6-thioguanine. This was done with the knowledge that advanced breast cancer has a small growth fraction and clinically probably represents the "plateau" phase of tumor growth.

MATERIALS AND METHODS

Thirty-one patients with advanced breast cancer were treated with the combination of vinblastine and 6-thioguanine between October 1976 and September 1977. All patients had a histologically confirmed diagnosis of advanced breast cancer and they all signed an informed consent document defining the risks and benefits of the treatment program. All patients had received prior chemotherapy with cyclophosphamide, vincristine, 5-fluorouracil, adriamycin, and prednisone. In addition, most patients had received methotrexate. Upon entering the study, all patients had documented progression of their disease defined as a 25% increase in measurable tumor over a period of ≥ 2 weeks. All patients in this study had evaluable disease, a wbc count $> 4000/mm^3$, and a platelet count $> 100,000/mm^3$.

Vinblastine was given on Day 1 at a dose of 5 mg/m^2 by iv push. 6-Thioguanine was given on Days 2–6 at a dose of 100 mg/m^2 orally divided into two daily doses. The dose was rounded to the nearest 40 mg. In patients who had had prior radiation therapy within 12 months to the pelvis, spine, or sternum, the first dose of vinblastine was 3 mg/m^2 and the first dose of 6-thioguanine was 70 mg/m^2, with escalation to 5 and 100 mg/m^2 respectively at the second course in the absence of significant hematologic toxicity. The sequence was repeated every 2 weeks provided the wbc count was $> 4000/mm^3$ and the platelet count was $> 100,000/mm^3$. If the wbc count was between 3000 and $4000/mm^3$ or if the

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platelet count was between 75,000 and 100,000/mm^3, 50% of the dosage was given, and when the counts fell below these levels, therapy was withheld and blood counts were checked weekly until drug administration could be resumed.

Clinical or radiographic measurements were used to define response. The response criteria used were those defined by the International Union Against Cancer (6).

The sites of evaluable disease were studied monthly in order to determine response. In those patients with bone disease, roentgenographic studies of indicator lesions were used in preference to repeat bone scan. In no patient was a pleural effusion or brain metastasis the only site of measurable disease. The clinical characteristics, results of prior therapy, and responses to the current program are presented in table 1.

RESULTS

One hundred and seventeen courses of medication were given to the 31 patients in this study. Eight patients received one course of medication only and six of these eight patients died within 2–4 weeks of the first course due to progressive disease. Two other patients with disease progression were changed to other therapy. A partial response was seen in two patients, one with malignant ascites and pleural effusions (histologically confirmed) and the other with bone, skin, and pleural metastases. Stabilization of disease was seen in an additional 12 patients. The duration of response of the two patients with partial remission was 2 and 6 months respectively.

Two of the 31 patients had a partial response for an objective response rate of 6% (95% confidence levels: > 0 and $< 17\%$). The 14 patients who had a partial response or stabilization had a median survival time of 23 weeks while the median survival time of those with continued progression was only 7 weeks.

The percentage of optimal dose administered and the resultant hematologic toxicity are defined in table 2. Hematologic toxicity was moderate but no patient died of drug-related sepsis or bleeding. No significant motor loss or paresthesias was noted in patients treated with this regimen and nausea and vomiting were minimal.

TABLE 1.—Patient data

	Response		
	Partial and stable (%)	Progression (%)	Totals (%)
No. of patients	14	17	31 (100)
Age in yrs (median)	59	57	57
> 50 yrs	12 (86)	13 (76)	25 (81)
Performance status			
Ambulatory	5 (36)	5 (29)	10 (32)
≤ 50% bedridden	4 (28)	5 (29)	9 (29)
> 50% bedridden	5 (36)	7 (41)	12 (39)
Disease-free interval < 2 yrs	8 (57)	8 (47)	16 (52)
Prior treatment			
Radiation	14 (100)	14 (82)	28 (90)
Hormonal	7 (50)	12 (70)	19 (61)
Response to prior chemotherapy	4 (28)	6 (35)	10 (32)
Distribution of metastases			
Soft tissue*	8 (57)	9 (53)	17 (55)
Pleura, lung parenchyma	7 (50)	7 (41)	14 (45)
Bone	9 (64)	11 (65)	20 (64)
Liver	2 (14)	6 (35)	8 (26)
Brain	1 (7)	0	1 (3)
Median survival time (wks)	23.0	7.0	14.5
	$P < 0.002$†		

* Includes skin, nodal, and subcutaneous metastases.
† Wilcoxon-Gehan tests.

TABLE 2.—Toxicity

	Course				
	1	2	3	5	Total*
No. of patients	31	23	17	9	31
% optimal dose					
Vinblastine	Full	81	80	82	
6-Thioguanine	Full	71	74	67	
Wbc count (cells/mm³)					
≥ 4000	18 of 31 patients (58%)	14 of 23 patients (61%)	11 of 17 patients (65%)	6 of 9 patients (67%)	77 of 117 courses (66%)
3000–3999	6	3	2	1	19 (16%)
2000–2999	6	5	2	1	15 (13%)
< 2000	1	1	2	1	6 (5%)
Platelet count (cells/mm³)					
> 100,000	27	20	16	9	109 of 117 courses (93%)
75,000–100,000	1	2	0	0	3 (3%)
20,000–75,000	2	1	1	0	4 (3%)
< 20,000	1	0	0	0	1 (1%)

* Includes all courses (117) given in the entire study.

DISCUSSION

The sequential use of vinblastine and 6-thioguanine in this study resulted in a very low objective response rate. However, almost half of the patients had disease stabilization on this program and fared better than those who progressed (table 1). All patients in this study had advanced disease which had become refractory to prior chemotherapy and in most cases hormonal therapy. In addition, 12 of the 31 patients had radiation therapy in the 12 months before the study. For these reasons, this group of patients represents a very poor-risk category and a high response rate would not be expected. Prior studies with vinblastine and 6-MP indicate that these drugs used as single agents have a response rate similar to our combination.

The observation that almost half of the patients in our study had stabilization of their disease with only moderate toxicity may indicate that there is a role for this or a similar regimen in patients with far-advanced disease refractory to conventional agents. It is likely that this type of sequential program would produce better results in untreated patients, but it is highly doubtful from these data whether the response rate would approach that of the currently used combination therapy in patients with advanced breast cancer.

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Phase II Trial of Maytansine in Patients With Advanced Colorectal Carcinoma [1,2]

Brief Reports and Preliminary Communications

Michael J. O'Connell,* Adi Shani, Joseph Rubin, and Charles G. Moertel [3]

Maytansine is an ansa macrolide compound which was chosen for clinical study because of its demonstrated mitosis-inhibiting properties in vitro (1) as well as its antitumor activity against model animal tumor systems including P388 leukemia, B16 melanoma, and Lewis lung carcinoma (2). An earlier phase I trial at our institution demonstrated tolerable clinical toxicity (3). The primary objective of this study was to assess the antitumor activity of maytansine in patients with advanced colorectal carcinoma.

PATIENTS AND METHODS

All patients had known colorectal adenocarcinoma with histologic proof of residual, recurrent, or metastatic disease. Each patient had at least one measurable site of known malignant disease to serve as an objective indicator of response to therapy. Hepatomegaly was employed as a measurable lesion if the liver had been proven to contain metastasis histologically and if there was a clearly palpable liver edge extending at least 5 cm below the xiphoid process or costal margin on quiet respiration. Radionuclide liver scans were used to assess response to therapy only if there was a clearly defined defect in isotope uptake measuring at least 5 cm in greatest diameter. Contraindication to patient selection included significant leukopenia (wbc count $< 4000/mm^3$) or thrombocytopenia (platelet count $< 130,000/mm^3$), major surgery within the preceding 3 weeks, radiation therapy or chemotherapy within the preceding 4 weeks (8 weeks for nitrosoureas), an active infectious process, total disability (Eastern Cooperative Oncology Group [ECOG] performance score of 4), or inadequate oral nutrition. None of the patients were clinically jaundiced, although several patients had elevations of SGOT or serum alkaline phosphatase and two patients had elevations of direct-reacting bilirubin ≤ 1.0 mg/100 ml.

The following studies were performed prior to each course of therapy: physical history, physical examination with measurement of indicator lesion(s), wbc and platelet counts, hemoglobin, chemistry profile including measurements of SGOT, serum alkaline phosphatase, direct and total bilirubin, and serum creatinine, and chest roentgenogram. Wbc and platelet counts were obtained weekly following the first two courses of therapy.

Maytansine was administered by rapid iv injection at a dose level of 0.6 mg/m^2/day for 3 consecutive days with treatment repeated at 3-week intervals for the first two cycles of therapy and every 4 weeks thereafter. Phenothiazines were administered as necessary to control nausea or vomiting.

Criteria for objective tumor response required at least a 50% decrease in the product of the bidimensional measurements of the most clearly measurable indicator lesion. If the liver was the primary indicator lesion, there must have been at least a 30% decrease in the sum of the linear measurements below the costal margins and xiphoid process. Progression was defined as a $> 25\%$ increase in the product of the perpendicular diameters of any indicator lesion, a $> 25\%$ increase in the sum of the distances below the costal margin and xiphoid process if hepatomegaly was the indicator lesion, the appearance of new lesions, the deterioration of performance status, or a decrease in body weight of $> 10\%$.

Thirty-one patients were entered in the study. The mean age was 63 years (range, 43–84 years). There were 17 men and 14 women. Sixteen patients had received no prior chemotherapy and 15 had previously received treatment with one or more cytotoxic agents. All patients were ambulatory and received outpatient chemotherapy (17 had an ECOG performance status of 0 or 1, and 14 had a performance status of 2 or 3). The site of the primary indicator lesion was the liver in 18 patients, the lung in nine, the abdomen in two, and other sites in two. The serum creatinine level was ≤ 1.6 mg/100 ml in all patients. The serum alkaline phosphatase level was elevated in 20 patients (more than twice

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TABLE 1.—Objective tumor status at 6 wks

	No. of patients with—		
	Regression	Stable disease	Progression
No prior chemotherapy	0	4	12
Prior chemotherapy	0	2	13
Totals	0	6	15

TABLE 2.—Toxic effects in 31 patients

	Degree (% of patients)	
Toxic effect	Mild to moderate	Severe
Nausea and/or vomiting	23	10
Diarrhea	39	6
Alopecia	13	—
Paresthesias	6	6
Myalgias	—	6
Paralytic ileus	—	3

the normal level in nine patients) and the SGOT level was elevated in 19 patients (more than twice the normal level in seven patients).

RESULTS

The objective tumor status at 6 weeks is reviewed in table 1. None of the patients in this study experienced an objective tumor regression.

Toxicity is reviewed in table 2. Vomiting and diarrhea, when observed, usually occurred at the end of the treatment cycle or within the first several days following therapy. Peripheral paresthesias were noted in four patients. Two patients developed severe muscular aches. Paralytic ileus occurred in one patient and responded to conservative management. All severe toxic effects except one were observed after the first or second treatment cycle. The data on cumulative toxicity are limited, however, since only six patients have received more than two cycles of therapy. The incidence of severe toxic effects appeared to be increased among patients with elevated SGOT or serum alkaline phosphatase levels although the number of patients experiencing severe toxicity is too small to permit a definitive conclusion. However, both patients with elevated direct-reacting serum bilirubin experienced severe neurologic toxic effects.

DISCUSSION

We were unable to demonstrate any antitumor effect of maytansine in our patients with advanced colorectal carcinoma. Although most patients tolerated treatment without experiencing serious side effects, severe vomiting, diarrhea, and neurologic toxic effects were occasionally observed.

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Reports From the Workshop on the Use of Steroids as Carriers of Cytotoxic Agents in Breast Cancer

Cancer Therapy Evaluation Program
Division of Cancer Treatment
National Cancer Institute

Organized by: Franco M. Muggia
Marc Lippman
Jean-Claude Heuson

July 15, 1977
Bethesda, Maryland

Introduction: Chemical Structures Considered for Clinical Use

F. M. Muggia [1,*]

In the past, a number of agents have been synthesized containing a steroid nucleus and a cytotoxic moiety. Much of this work preceded the knowledge of specific receptors and was stimulated by the introduction of phenesterin into clinical trials in the US (1) following preliminary findings of antitumor activity in the Soviet Union (2). Other agents were subsequently synthesized with the aim of coupling hormones and cytotoxic agents for their possible usefulness in hormonally responsive cancers. Animal tumor data on many of these agents are available (3). Estradiol mustard was advanced into clinical trial and had a relatively short phase I and II study by the Eastern Cooperative Oncology Group (4). The agent had associated hormonal effects of estrogen and the toxic effects of nitrogen mustard derivatives, suggesting uncoupling of the two effects. The clinical activity was not striking. Coupling of similar moieties to testosterone and to dehydroepiandrosterone did not advance beyond large animal toxicology studies; both of these compounds exhibited hematologic toxic effects and were erratically absorbed orally (data on file at the Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute).

Other compounds such as estramustine (Estracyt) and phosphate prednimustine, introduced recently in Europe, and their clinical trials will be discussed further by Dr. Catane elsewhere in this issue (*see* Summary of Other Presentations Pertaining to Cytotoxic Agents Conjugated to Steroids). Once again, evidence for receptor affinity did not play a part in their synthesis or introduction into clinical testing. Additional suggestions for other carriers for alkylating moieties have recently been made by Sieber and Adamson (5).

Other compounds which have been synthesized, but have received only very limited or no testing in biologic systems, will be presented by Drs. Blickenstaff, Hamacher, and van Lier in their reports in this issue. Some of the principles of synthesis of such compounds in relation to retention of their receptor specificity will be outlined by Dr. Katzenellenbogen in his report. Preliminary exploration of sites of alkylation of these compounds will be presented in Dr. Warren's report.

Important questions beyond the synthesis of these compounds were raised by others who participated in this workshop and in the general discussion. Major questions related to their mechanism of action, our methods of assaying their receptor specificity, and the assessment of antitumor activity. These issues were addressed by several participants, and conclusions from presentations and discussions are summarized in the final section of these reports.

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Comparative Binding Affinities of Estrogen Derivatives [1]

John A. Katzenellenbogen [2,*]

Many steroid derivatives have been prepared. In most cases, the object of the chemical alteration of the parent steroid has been the attainment of a desired level or character of biologic activity. More recently, steroid derivatives that are designed to act as functional agents have been prepared. It is convenient to term the first type of derivatives "hormonal derivatives," while the second type may be called "steroid reagents," a term that emphasizes their functional nature. This review will focus on derivatives of the second type and will cover work both from this laboratory and from other laboratories, with major emphasis on the estrogens.

The Functional Nature of Steroid Reagents: Receptor and Nonreceptor Interactions

Steroid reagents are dual-purpose or, in a sense, bifunctional compounds. They are designed to interact with the receptor, but their purpose is not to elicit a hormonal response; rather, their principal function is usually completely independent of this interaction. Examples of functions to be achieved with these reagents are listed in table 1.

TABLE 1.—Steroid reagents and function

Reagent	Function
Affinity label	Covalent attachment
Affinity chromatography	Immobilization
Radiopharmaceuticals	Gamma-ray emission
Fluorescent probe	Fluorescence
Drug	Drug carrier

A useful concept to develop deals with the relationship between the functional portion of the reagent and the portion that is involved in receptor interaction. Two limiting cases can be considered (fig 1). In the first case, the portion of the molecule involved in the receptor interaction (steroid) and the functional unit are completely separated. Reagent preparation will generally involve connection of the two portions; therefore, this type of reagent may be termed "conjugated" or "connected." In the opposite extreme are reagents in which the functional element is congruent or wholly contained within the structure of the steroidal ligand; these are "integrated reagents." It is possible, of course, to have reagents in-between these two extremes, that is, "partially integrated reagents."

The two keynotes of steroid receptor interaction are high affinity and stereospecificity. Therefore, an obvious concern in the design of steroid reagents is how chemical modification of the steroid will affect its receptor binding affinity. Often, information can be gleaned from structure-activity relationships that suggests which portions of the steroid ligand can be altered without severe consequences. More recent data relating structure to receptor binding affinity speak more directly to this issue. In the concern for maintaining high receptor binding affinity, however, one must be careful not to overlook two other interactions that may prove crucial to the

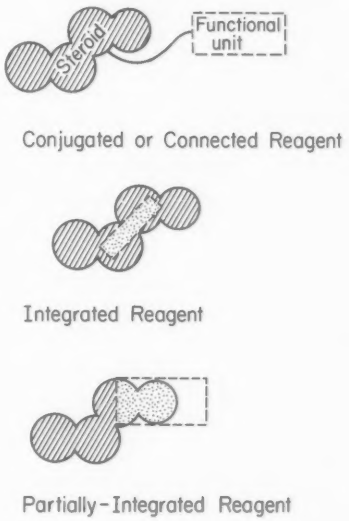

FIGURE 1.—Classes of steroid reagents.

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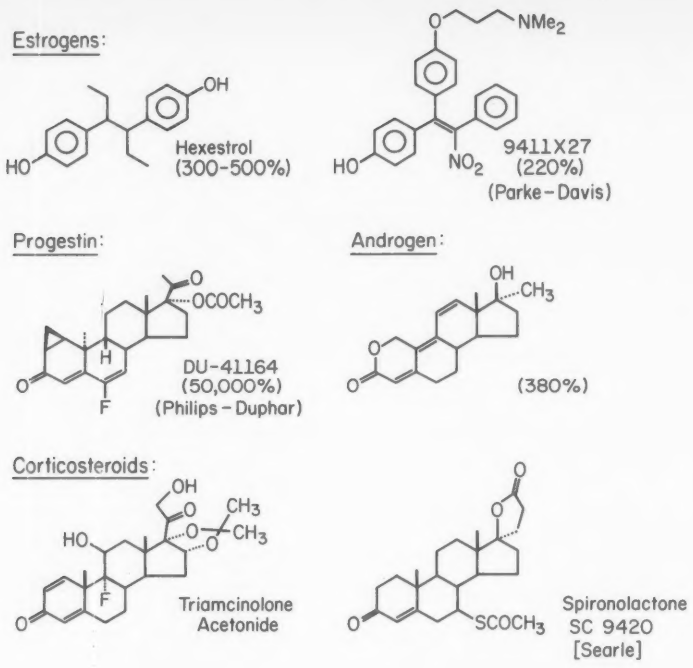


FIGURE 2.—High-affinity ligands for steroid receptors. % figures in parentheses = association constants relative to that of the normal endogenous ligand, estradiol, progesterone, dihydrotestosterone, and cortisol, respectively. Binding affinity data are from: hexestrol (1), 9411X27 (2), DU-41164 (3), and the androgen (4).

ultimate utility of the steroid reagent. Alteration of ligand structure can affect the extent to which the reagent will interact with nonreceptor proteins (nonspecific binding). Also, structure modification can change binding selectivity, that is, the derivative may have increased affinity for the receptors for other steroids. An example of these types of complications will be given later.

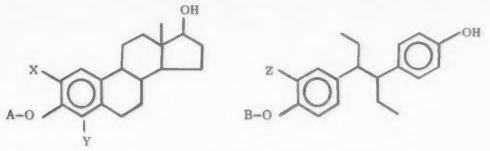
Ligands With Particularly High Affinity for Steroid Receptors: Tolerance Towards Substitution

In designing a steroid reagent for a particular receptor, one might assume that the parent or endogenous steroid would be the best starting point for chemical modification. This may not be the case, for two reasons. First, the parent steroid is often not the highest affinity ligand for the receptor. In fact, for all of the steroid receptors, ligands are known with considerably higher affinity than the parent steroid. Some of these are listed in figure 2. Second, in some cases, introduction of a particular substituent causes a greater relative decrease in the receptor binding of the parent steroid than of the ligand analog.

As is indicated in table 2, we have observed two types of behavior in our studies on steroidal and nonsteroidal estrogen derivatives (5). Parallel modifications have been made in estradiol and in hexestrol. In some cases, the structural alteration causes parallel changes in affinity. For example, methylation of estradiol and hexestrol causes equivalent decreases in binding affinity of both species. In contrast, the larger carbethoxymethyl ether is tolerated more favorably by hexestrol. An ortho nitro group is well-tolerated by hexestrol and at steroid position C–4, but is poorly tolerated at steroid position C–2. The increased tolerance of hexestrol towards ortho substitution, however, is particularly evident in the azide and the iodo derivatives (5,6).

We have suggested that the greater tolerance of hexestrol towards these sorts of substitution derives from its symmetry and conformational flexibility (5). Ortho monosubstitution of *meso*-hexestrol produces a racemic mixture of enantiomers, one of which can bind with its substituent in a position congruent with either a C–2 or C–4 substituted steroidal ligand,

TABLE 2.—Relative effects of substituents on receptor binding of steroidal vs nonsteroidal estrogens*



	Relative binding affinity†	
	Estradiol	Hexestrol
	A	*B*
H	100	100
CH_3	3.0	6.5
$EtOCOCH_2$	0.034	0.54
	X	*Y*	*Z*
H	100	100	100
NO_2	< 0.03	6.2	5
N_3	3	0.9	24
I	< 0.03	< 0.03	14

* Data from ref 5.

† Binding affinities are measured by charcoal adsorption in a competition assay with [^{3}H]estradiol (5). Nos. = % K_a relative to that of the unfunctionalized ligand.

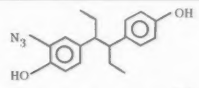
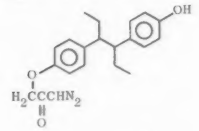
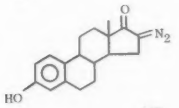
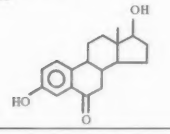
and the other which can bind at sites roughly equivalent to steroid positions C–15 and C–17. Thus, while a group on the steroid is positioned unambiguously within the receptor binding site, the symmetry and flexibility of hexestrol provide several alternative modes of binding for the group.

Estrogen Reagents

Photoaffinity labels.—We have prepared a number of derivatives of hexestrol and steroidal estrogens as photoaffinity labels for the uterine estrogen receptor (table 3). All of these reagents would be classified as partially integrated, except the second, in which the photoreactive chromophore (a diazocarbonyl group) is totally separate from the ligand portion of the molecule. With each compound, we have determined the reversible binding affinity to receptor (5). We have also assayed irreversible reaction with receptors in two ways. With unlabeled compounds, we determine the effectiveness of these reagents as receptor inactivators; after irradiation of the reagent-receptor complex, the surviving binding activity is measured by exchange with estradiol (2,7). As seen in table 3, there is no correlation between ligand binding affinity and inactivation efficiency, as these are two independent parameters. When the reagent is available in radiolabeled form, covalent incorporation can be measured directly (1,8). Differences between the attachment efficiency and inactivation efficiency indicate that some receptor inactivation occurs by a mechanism that does not involve the formation of covalent links between reagent and receptor.

Immobilized estrogens.—Table 4 presents structures of some estrogen derivatives that have been or might be used in receptor purification by affinity

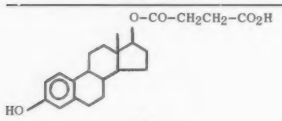
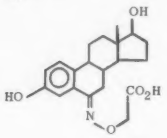
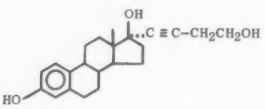
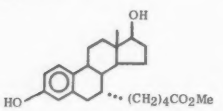
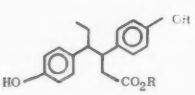
TABLE 3.—Estrogen photoaffinity labels*

Compound	Relative binding affinity (E_2 = 100)	Inactivation efficiency (%)	Attachment efficiency (%)
	70	15	15
	3	15	0
	0.5	22	(0)
	20	Linear	0†

* Data from refs 1 and 2.

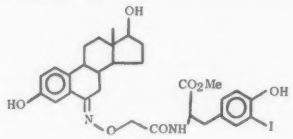
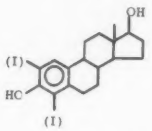
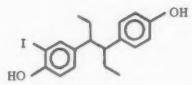
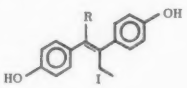
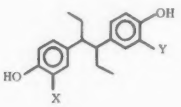
† This compound is an efficient covalent labeling reagent for albumins such as rat alpha-fetoprotein and serum albumin (J. A. Katzenellenbogen, R. J. Neeley, D. W. Payne, et al, unpublished data).

TABLE 4.—Estrogens useful for affinity chromatography

Compound		Relative binding affinity (E_2 = 100)
		0.3*
		0.002*
		0.5†
		5†
	R = CH_3	90‡
	R = nC_5H_{11}	10

* Data from ref 9.
† Data from ref 10.
‡ J. A. Katzenellenbogen and D. F. Heiman, unpublished data.

TABLE 5.—Gamma-emitting estrogens

Compound		Relative binding affinity (E_2 = 100)
		< 0.03*
		Very low*
		40†
	R = H R = CH_3 R = C_2H_5	0.3‡ 3.0 25
	Y = F, Y = H	103§
	X,Y = F	9

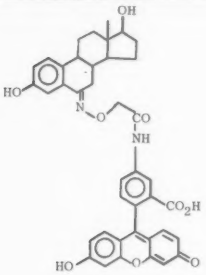
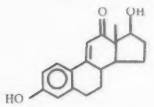
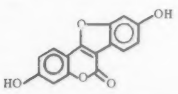
* Data from ref 11.
† Data from refs 6 and 11.
‡ J. A. Katzenellenbogen and K. J. Allison, unpublished data.
§ J. A. Katzenellenbogen and D. F. Heiman, unpublished data.

chromatography. The first two are actually derivatives that are used in the preparation of estrogen antibodies, and they have a relatively low binding affinity for receptors. The fourth is functionalized at steroid C-7α, a site that appears to be quite tolerant of bulk. The final entry in table 4 shows two members of a series of hexestrol derivatives we have recently prepared that are functionalized at the end of the hexane chain. Both the methyl and pentyl esters have good binding affinity, suggesting that long-chain substitution at this site may also be well-tolerated.

Gamma-emitting estrogens.—Table 5 shows the structures of some steroidal and nonsteroidal estrogens that were prepared as potential radiopharmaceuticals for the selective detection of estrogen receptor-containing mammary adenocarcinoma. The first member is actually an iodinated ligand used in estrogen radioimmunoassay and has negligible receptor binding affinity. The ortho iodinated derivatives were mentioned previously (table 2) as examples of the greater tolerance of the hexestrol skeleton toward ortho substituents. Derivatives in the next series represent further integration of iodine into the ligand structure, in that iodine has replaced one of the ethyl substituents. The last member of this series, an analog of diethylstilbestrol, may have considerable promise as an in vivo diagnostic agent. Finally, the fluorinated estrogens, that could potentially be labeled with fluorine-18, also have high binding affinities.

Fluorescent estrogens.—Compounds that may be useful as fluorescent probes of estrogen receptor interactions are shown in table 6. The first member, prepared by Levison and Dandliker et al (12,13), is a classic example of a reagent of the conjugated type. Although the fluorescein unit has excellent fluorescent characteristics, the reagent itself has relatively low binding affinity. 9(11)-Dehydro-12-oxoestradiol, which we have prepared as a fluorescent probe, is an example of a nearly completely integrated reagent. This derivative has substantial receptor binding affinity and interesting fluorescent properties. Its fluorescence lifetime is too short for depo-

TABLE 6.—Fluorescent estrogens

Compound	Relative binding affinity (E_2 = 100)
	< 5*
	10–15†,‡
	20§

* Data from refs 12 and 13.

†J. A. Katzenellenbogen and R. J. Neeley, unpublished data.

‡	Acid	Base
λ max abs.	331 nm (17,700)	385 nm (23,700)
λ max excit.	353 nm	395 nm
λ max emiss.	440 nm [1]	474 nm [12]
τ	—	0.75 nsec
ϕ	0.03	0.57

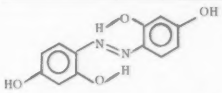
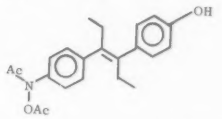
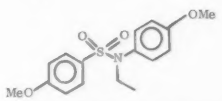
(ϕ is very sensitive to solvent dielectric)

§ Data from ref 14.

larization studies, but its strong absorbance and high quantum yield indicate that it may be a useful probe for receptor studies. The fluorescent phytoestrogen coumestrol has been studied by Lee et al (14); it too has high affinity for the receptor.

Other estrogen reagents.—Table 7 lists some other unusual steroid reagents. Azoresorcinol was prepared in 1953 by Urushibara and Takahashi (15) and was demonstrated to have substantial estrogenic activity. We have resynthesized this compound and the related tri- and di-hydroxy analogs and have measured their receptor binding affinity and mutagenic activity. These compounds, as well as the next member, an electrophilic stilbestrol analog (16), might be interesting reagents to probe the relationship between estrogen receptor binding and estrogen-induced tumorigenesis. Finally, the sulfonamide prepared by Osawa (17) has both antibiotic and estrogenic activity.

TABLE 7.—Other estrogen reagents

Compound	Relative binding affinity (RBA) (E_2 = 100) and other properties
	RBA = 2%*; weakly uterotrophic (1/100)†; mutagenic (50 rev/nmol in TA 100–3)*
	Weakly estrogenic (1/500)‡
	Weakly estrogenic (1/50)§; antifungal, antibiotic

*J. A. Katzenellenbogen and D. F. Heiman, unpublished data. Mutagenic activity was assayed in Ames System with *Salmonella* strain TA 100-3 either with or without microsomal activation.

†Data from ref 15.

‡Data from ref 16.

§Data from ref 17.

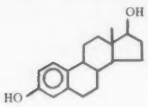
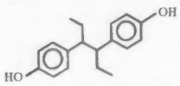
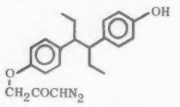
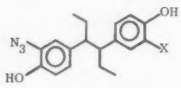
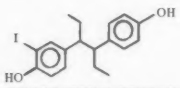
Nonspecific Binding

At the beginning of this report, it was mentioned that structural modification of steroid ligands can have consequences in terms of interaction of the altered ligand both with receptor and with nonreceptor binding sites. This situation has actually been encountered in our work on estrogen radiopharmaceuticals (6).

On the basis of our studies on the preparation of photosensitive estrogens (5), we had come to recognize that the ortho position of hexestrol was much more tolerant of large substituents than was estradiol (*see* table 2). Therefore, we prepared iodohexestrol as a potential gamma-emitting estrogen radiopharmaceutical. We were pleased but not surprised to find that its affinity for the estrogen receptor from rat uterus, measured by competition assay, was indeed high (table 5). However, once radiolabeled iodohexestrol had been prepared, it became apparent that introduction of the iodine substituent had also elevated the affinity of this reagent for nonreceptor proteins to such an extent that estrogen-specific binding could not be measured directly in uterine cytosol preparations.

The sucrose gradient pattern, shown in figure 3, indicates the extent of nonreceptor binding (4S peak; fractions 8–14) of this compound compared to the receptor binding (8S peak; fractions 16–23); in contrast, the binding of estradiol is highly selective for the receptor. Despite its reasonable affinity for

TABLE 8.—Relative nonspecific binding of estrogen reagents estimated by Hansch analysis

Compound		Relative nonspecific binding affinity*
		1
		6.6
		11
	X = H X = N_3	15 32
		46

*Calculated from equation 1, using substituent coefficient values from ref 19 and sources cited therein.

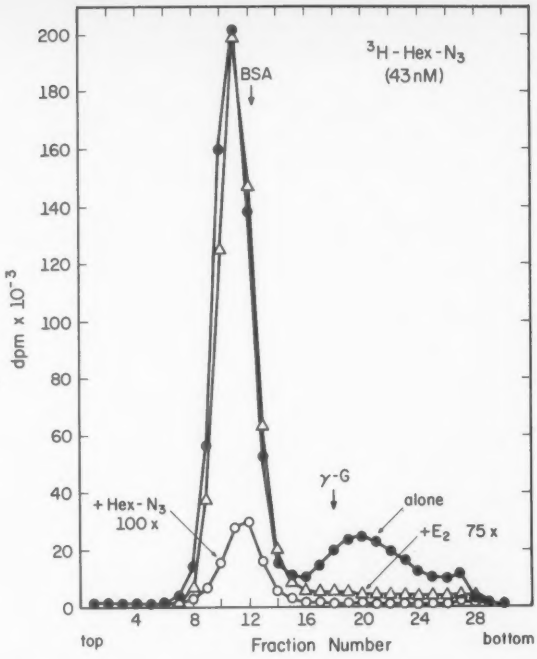


FIGURE 3.—Sucrose density gradient centrifugation analysis of [^{3}H]estradiol ([^{3}H]E_2) and [^{3}H]-iodohexestrol ([^{3}H]-I-Hex) binding in rat uterine cytosol. Samples of rat uterine cytosol (200 μl; 0.56 uterine equivalent) were incubated with the indicated concentration of [^{3}H]E_2 or [^{3}H]-I-Hex for 5 hrs at 0°C, charcoal-dextran treated, and then layered onto 3.6-ml 5%–20% linear sucrose density gradients and centrifuged at 246,000 × *g* for 13 hrs. Gradients were fractionated from the top by displacement with 50% sucrose, and 3-drop fractions were collected for scintillation counting. Carbon-14 labeled bovine serum albumin (4.7S) and γ-globulin were included as markers in some runs (*from* ref 6).

receptor, the very high level of nonspecific binding made this compound totally unsatisfactory for its intended use as a radiopharmaceutical selective for estrogen receptor-containing mammary adenocarcinoma.

Pharmacologists have long known that increased lipophilicity generally translates into increased nonspecific binding. In fact, an empirically based quantitative statement of this relationship has been provided by Hansch (18) and can be used as a predictor of the degree of nonspecific binding of steroid reagents. Hansch has reported that in a series of lipophilic molecules, the reciprocal of the concentration (1/C, related to the association constant) required to achieve a 1:1 stoichiometry in complexing to bovine serum albumin is related to the 1-octanol:water partition coefficient of the compounds (P) by the following equation:

$$\log (1/C) = 0.75 \log P + 2.3. \quad [1]$$

This equation indicates that a tenfold increase in the partition coefficient translates into about a sevenfold increase in nonspecific binding. Since bovine serum albumin is probably a good model for nonspecific binding, it is quite likely that this relationship will also hold for the interaction of steroid reagents with nonspecific binding sites. The Hansch approach is particularly useful because from studies on model compounds, Hansch has derived a set of coefficients that represent the contribution of various substituents to the partition coefficient (19). Using these substituent coefficients and the relationship in equation 1, one can calculate the predicted level of nonspecific binding of a number of steroid reagents (20). Examples are given in table 8. In a number of instances, we have experimentally verified the calculated values of log P, and from sucrose gradient and other binding analyses, it is clear that those derivatives that have high values for log P are the ones that suffer most from high nonspecific binding.

CONCLUSION

A wide variety of steroid reagents, ranging from ones that are simply conjugates between a steroid and a functional unit to ones in which the functional

unit is nearly perfectly integrated within the ligand, have been prepared in recent years. While these compounds are already of interest and utility, the formulation of conceptual guidelines for the design of such reagents and the increasing availability of data relating structure and binding affinity will assist in the preparation of new reagents with both high affinity for their intended receptor sites and minimum affinity for other sites. This portends favorably for the importance of steroid reagents in the future.

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Steroid-Nucleosides [1]

J. E. van Lier,* G. Kan, D. Autenrieth, and E. Hulsinga [2]

SUMMARY

A new class of potential chemotherapeutic agents consisting of steroids coupled with nucleoside bases is described. Such structures may be viewed as nucleosides in which the sugar moiety is replaced by a steroid molecule. We may therefore expect the nucleoside characteristics to give interference at the DNA level, resulting in the desired antitumor activity, whereas the steroid moiety may provide target specificity. A number of such coupling products were prepared and preliminary biologic studies are reported.

[Cancer Treat Rep 62:1251–1253, 1978]

Most anticancer chemotherapeutic drugs have undesirable side effects due to their nonselective inhibition of both normal and tumor cell proliferation, and must be given at high concentrations to eradicate all growing tumor cells. With a view to reducing the required doses of antitumor agents, attempts have been made to increase their specificity and cell-penetrating properties by coupling them with steroid hormones (1,2). In particular, steroidal alkylating agents have been studied and their carcinostatic properties, if they are used in combination with other modes of therapy, have been reported (3). In addition, they have been evaluated at the biochemical level for properties judged essential for organ-specific activity, eg, affinity for steroid receptors and the in vivo stability of the linkage between the hormone and the alkylating moiety (4,5).

We have recently initiated a program on the synthesis of a new class of agents by coupling steroids with naturally occurring pyrimidine bases via a C–N linkage and have conducted preliminary studies of their toxicity and antitumor activities (6). The structures may be regarded as analogs both of steroid hormones and of nucleosides in which the sugar moiety has been replaced. As with steroids coupled with cytotoxic agents, our drugs may exhibit organ specificity in those instances where affinity for the receptor of the steroid molecule is retained. However, they differ from such combination drugs in that neither of the components alone exhibits cytotoxic properties. Instead, antitumor activities are anticipated only upon fusion of the two principal entities. Their resemblance to nucleosides may lead to interference at the DNA replication level, to give the desired antitumor activity, while the steroid moiety may give target specificity, permitting reduction of the required dose levels. Furthermore, our steroid-nucleosides are less likely to have adverse side effects than conventional drugs since their degradation products are metabolites of natural steroids and nucleoside bases.

Synthesis

Coupling between steroids and nucleoside bases was accomplished by adapting general methods used in nucleoside synthesis (7,8) and by methods developed in our own laboratory. Briefly, the appropriate hydroxysteroid was converted to the bromo- or mesyl-derivative and reacted with the persilylated heterocyclic in the presence of an $HgO/HgBr_2$ or Friedel-Crafts ($SnCl_4$) catalyst to give the C–N linkage. In this manner, we obtained sufficient amounts of material to allow structure elucidation and some biologic testing of the thymine, uracil, and 5-fluorouracil derivatives at position 3 of cholest-5-ene (Ia) and pregn-5-en-20-one (Ib), and position 21 of progesterone (IIa), 21-desoxycortisone (IIb), and 11-ketoprogesterone (IIc) (fig 1). Substitution of the 3-bromosteroids with persilylated pyrimidines gave

[1] Supported by grant MA-4051 from the Medical Research Council of Canada.

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mixtures of the four possible isomers, N_1-3α, N_1-3β, N_3-3α, and N_3-3β. In addition to 3-substituted products, imidazole-related heterocyclics gave 3,5-cyclo, 6-substituted steroids. The 21-bromosteroids gave rise to mixtures of two isomers only, N_1- and N_3-substitution. Enrichment of specific coupling products in the reaction mixtures could be influenced by the solvent, the type of catalyst, and the nature of the heterocyclic base. Details of these procedures will be presented elsewhere.

Biologic Testing

A number of our Type I and II steroid-nucleosides were tested for acute toxicity on CD-1 outbred albino female mice at 200 mg/kg (given ip in 0.2 ml of sesame oil). None of these compounds proved to be toxic at these levels, with the exception of the 3α-imidazole derivative of pregn-5-en-20-one (6). A series of thymine and uracil derivatives with differences in the site of attachment to the base and variation in the steroid moiety (Ia,b; IIa) were tested in Fischer 344/CRBL rats bearing the 13762 mammary adenocarcinoma, which is sensitive to estrogens and androgens but not to progesterone (9). Accordingly, it is unlikely that any effect on the lifespan of the treated animals could result from breakdown of the drugs to give the free steroid. Treatment was started 24 hours after animals were given subcutaneous tumor grafts. The agents were administered sc in sesame oil (10 mg/ml) at a dose of 10 mg/kg on alternate days for a total period of 18 days. Control animals treated with sesame oil alone developed large tumors and had a mean survival of 29.1 days (ten animals per group). The lifespan was increased for rats treated with one of the isomeric 3-uracil derivatives of pregn-5-en-20-one (Ia, R_1 = H [NSC-281832]; T/C, 167%) and to a lesser extent, with the 21-thymine derivative of progesterone (IIa, R_1 = H [NSC-281830]; T/C, 156%). Treatment with the 3α-imidazole derivative of pregn-5-en-20-one also prolonged the survival (T/C, 140%) (6).

A group of 16 different Type I and II steroid-nucleosides (NSC Nos. 281828–281843), including the thymine, uracil, and 5-fluorouracil N_1- and N_3-derivatives of 21-desoxycortisone (IIb), 11-ketoprogesterone (IIc), and the steroid-nucleosides used on the 13762 system (Ia,b; IIa), were screened against the L1210 lymphoid leukemia in female mice through the courtesy of the screening program of the National Institutes of Health, Bethesda, Md. Products were injected ip at different dose levels, and were suspended in Klucel (hydroxypropylcellulose), on Days 1, 5, and 9 after tumor implantation. Only one product, the 3-uracil derivative of pregn-5-en-20-one (NSC-281832), gave presumptive activity (T/C, 130% at 200 mg/kg).

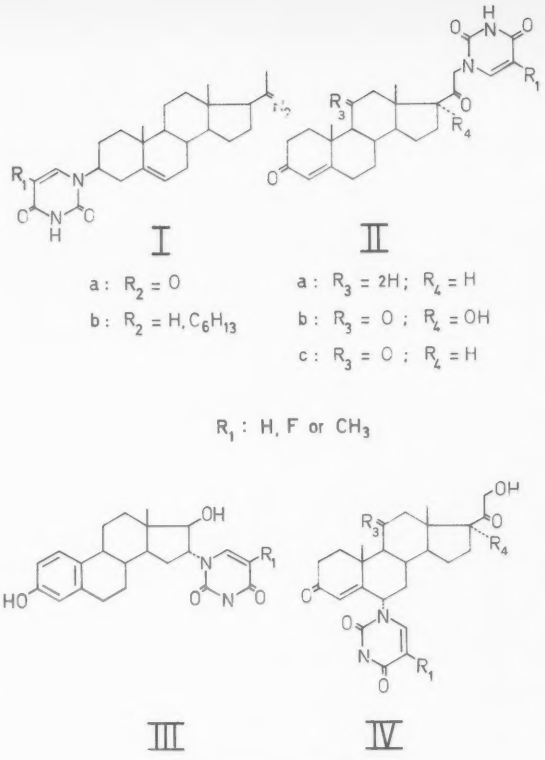

FIGURE 1.—Steroid-nucleosides.

The same 16 products were evaluated for their steroid-receptor binding properties in tissue culture cell lines which contain receptors for estrogen, androgen, progesterone, and glucocorticoid. The 21-substituted progesterone derivatives showed affinity for androgen receptor whereas some of the 21-substituted corticoids exhibited affinity for glucocorticoid receptor. None of these 3- or 21-substituted steroids showed significant binding to estrogen or progesterone receptor (10).

DISCUSSION

In our first approach to the synthesis of steroid-nucleosides, we chose to couple pyrimidines, purines, and some imidazole-derived heterocyclics with steroids by substituting the 3-hydroxyl group of cholesterol and pregnenolone (Type I) and the 21-hydroxyl group of 21-hydroxyprogesterone and

some corticoids (Type II). The two moieties are coupled via a C–N linkage rather than an ester bond in order to ensure in vivo stability of the complex. At least one of the isomeric 3-uracil derivatives of pregn-5-en-20-one (Ia, NSC-281832) showed presumptive antitumor activity in two different mammalian tumor systems, although the Type I products show little affinity for steroid receptors. This may indicate that certain steroid-nucleosides can function as analogs of nucleosides or as analogs of the pyrimidine base. Such characteristics are known to provide interference at the DNA replication level, or to inhibit vital enzyme functions such as thymidylate synthetase and dehydrofolate reductase.

In order to determine their effect at the biochemical level, we are preparing the ^{3}H-^{14}C-labeled analogs of our active products. We are also pursuing the synthesis of steroid-nucleosides which are more likely to retain a high affinity for specific steroid receptors with a view to combining antitumor activity with target specificity. In particular, C_{16}- and C_{6}-substituted steroids (Type III and IV) appear attractive since pyrimidine attachment at these positions leaves all the functional groups of the steroid molecule exposed for interaction with binding sites in the target tissue.

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Biochemical Assays for the Selection of Cytotoxic-Linked Estrogens With Potential Therapeutic Activity[1]

G. Leclercq* and J.-C. Heuson[2,3]

The interest in using estrogens linked with cytotoxic agents relies on the principle that such drugs might bind to estrogen receptors (ER) in target tissues, including breast cancers, and would thereby specifically concentrate into these tissues. To reach this goal, some conditions must be fulfilled. Among them, the drugs should have a significant binding affinity for ER and not be easily degraded outside the cancer cells into their main moieties: hormones and cytotoxic agents. In the past, biochemical assays have been used in our laboratory to evaluate whether a drug will fit these criteria. These assays require only small amounts of drug (1–10 mg) which need not be radioactively labeled. They are much simpler, cheaper, and faster than classic pharmacologic assays and appear therefore especially adequate for screening large numbers of compounds.

EVALUATION OF THE BINDING ABILITY OF A DRUG AND ITS STABILITY IN TISSUES AND BIOLOGIC FLUIDS

Binding Ability to ERs

Three assays were used for establishing the existence of a high-affinity interaction between a drug and ER. They were performed with cytosol preparations from immature rat uterus, dimethylbenzanthracene-induced rat mammary tumors, or human breast cancers. Since the detailed experimental conditions have previously been described (1,2), only general principles will be given here.

The apparent binding affinity of the drug was measured by competitive inhibition of the binding of ^{3}H-estradiol-17β (^{3}H-E_2). The cytosol was incubated with a saturating concentration of ^{3}H-E_2 in the absence or presence of increasing amounts of drug or unlabeled E_2. The relative concentration of the drug and of unlabeled E_2 required to achieve 50% inhibition of the binding of ^{3}H-E_2 gives its apparent binding affinity. The reversibility of drug binding was assessed by evaluating its displacement from the receptor sites by ^{3}H-E_2. The absence of displacement is indicative of irreversibility of binding. The degradation of the drug into one or several compounds with higher binding affinity was analyzed by a time-course study of its ability to inhibit the binding of ^{3}H-E_2. A progressive increase of inhibition is often indicative of the breakage of the link between the estrogen and the cytotoxic group.

Stability Outside the Target Tissues

The stability of a drug outside the target tissues was studied by incubation experiments in the presence of serum or liver extracts. The drug at a concentration producing only a small inhibition of ^{3}H-E_2 binding was preincubated in serum or liver extracts. The amount of high-affinity compound(s) produced was then measured by a competitive binding assay after mixing the preincubated solution with uterine cytosol added with ^{3}H-E_2.

MAIN PROPERTIES OF NINE ESTROGEN-NITROGEN MUSTARD DERIVATIVES

The binding affinity for ER of nine estrogen-nitrogen mustard derivatives was measured. These compounds are classified in figure 1 according to the extent of substitution by the cytotoxic agents of the two oxygen functions of their estrogen moieties (hydroxyl groups for E_2, diethylstilbestrol, or hexestrol; hydroxyl and cetonic groups for estrone). In class A the two functions are substituted, in class B only one is substituted, and in class C none is substituted. This classification was based on the fact that these groups in the free state are required for maximum binding to ER.

All drugs in groups A and B were characterized by a very low apparent binding affinity for ER (roughly 1000–10,000 times lower than E_2). In fact,

[1]Supported by contract N01-CM-53840 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, and by a grant from the "Fonds de la Caisse Générale d'Epargne et de Retraite."

[2]Service de Médecine et Laboratoire d'Investigation Clinique, Institut Jules Bordet, Brussels, Belgium.

[3]We wish to thank Dr. H. Hamacher (University of Tübingen, Germany), Dr. O. Yoder and R. B. Ing (National Cancer Institute, Bethesda, Md), Dr. I. Konyves (A. B. Leo, Helsingborg, Sweden), and Dr. D. N. Richardson (ICI, Macclesfield, United Kingdom) for providing the compounds used in this work.

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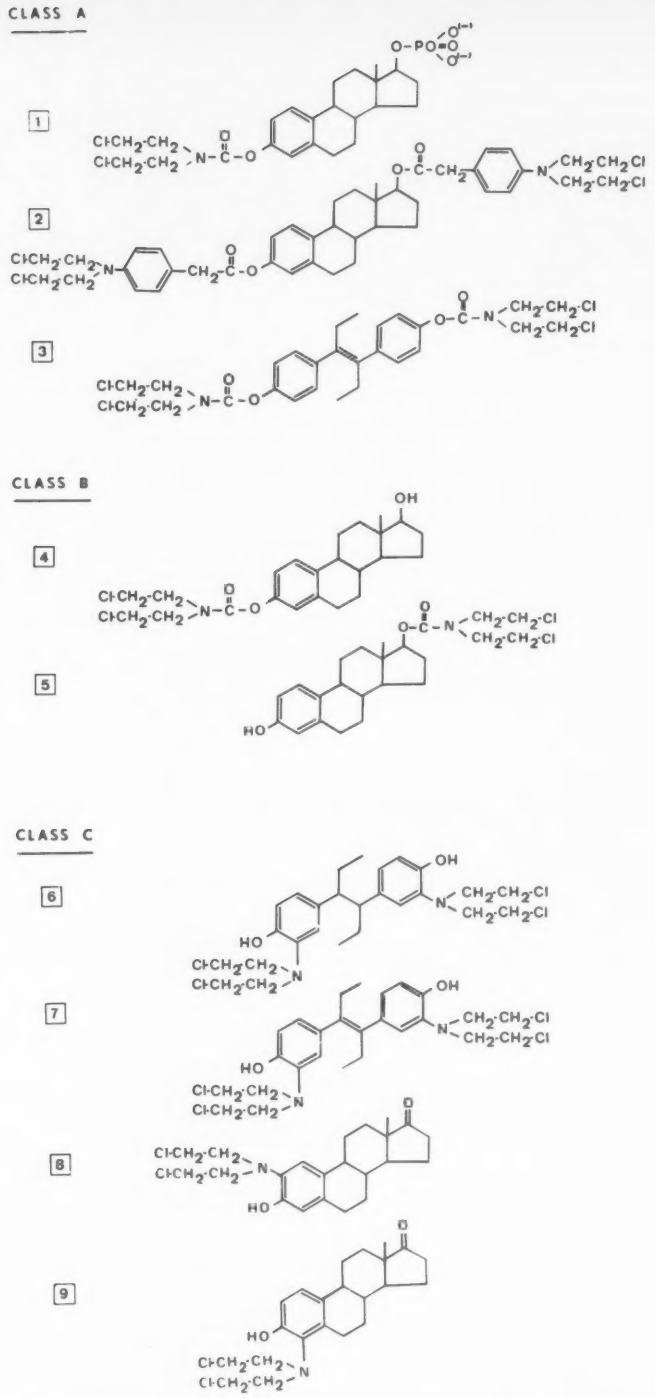

FIGURE 1.—Name and/or code No. of drugs: 1 = Estracyt, Leo 299; 2 = estradiol mustard; 3 = ICI 85966; 4 = dephosphorylated Estracyt, Leo 275; 5 = Leo 298; 6–9 = drugs produced by Dr. H. Hamacher (Ha IV, V, VI, and VII, respectively).

our results do not unequivocally establish that they really bind to the receptors. Indeed, in competitive assays using a 10,000–100,000-fold excess of drug, a contamination of 0.01%–0.001% of free estrogen would produce the same inhibitory effect. On the other hand, time-course studies revealed that E_2 derivatives were progressively metabolized into one or several compounds with higher affinity. Although the chemical nature of the latter was not established, free E_2 was very likely produced because the metabolites were displaced from the receptor sites by ^3H-E_2 at a rate close to that of unlabeled E_2. Of the drugs in class C, compounds 6 and 7 were also characterized by a very low apparent binding affinity (10,000 times lower than E_2). The binding of these compounds was, however, irreversible, indicating that the compounds themselves were involved in the binding, rather than degradation products or contaminants. Drugs 8 and 9 were found to bind firmly (affinity ~30 times lower than estrone). Preliminary data suggested that the binding was irreversible under certain conditions. With regard to the drug's stability in serum and liver extracts, it was found that compound 1 (Estracyt) was rapidly degraded into compounds with higher binding affinity probably including E_2. Compound 4 (dephosphorylated Estracyt) was more stable under similar experimental conditions and seemed therefore more interesting from the therapeutic point of view.

From the present results, it appears that compounds 8 and 9 could be of therapeutic value only through a mechanism involving ER. In contrast to all other drugs, they bind significantly and probably in an irreversible fashion to the receptors. The great difference in apparent binding affinity between these drugs and the others of the same class (Nos. 6 and 7) seems to suggest that significant binding to the receptors requires that at least one of the oxygen functions of the estrogen moiety be free of interaction with the cytotoxic moiety. Linkage in positions other than and far from these oxygen functions should therefore be recommended for future syntheses.

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Summary of Additional Presentations From the Workshop on the Use of Steroids as Carriers of Cytotoxic Agents in Breast Cancer

Marc Lippman [1,*]

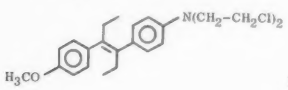
In order to provide a more complete picture of the information presented at this workshop, brief summaries provided by the participants of their own data are collected below. These short summaries may help the reader in appreciating previous accomplishments in the field as well as suggesting future directions.

Use of Steroids as Carriers of Cytotoxic Agents in Breast Cancer[2]

H. Hamacher, University of Tübingen, West Germany

Estrogens Linked With Nitrogen Mustard Groups

Based on (*a*) the fact that a majority of mammary carcinomas contain specific estrogen receptors, (*b*) the critical role of receptors in hormone action, and (*c*) the data on the relationship between molecular structure and the affinity of estrophilic compounds to the cytoplasmic hormone receptor, some potential mammotropic antineoplastic agents have been synthesized by binding nitrogen mustard groups to estrophilic carriers.



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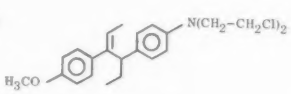

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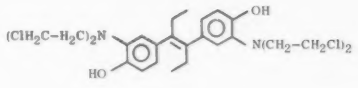
Compounds *1* and *2* are derived, respectively, from diethylstilbestrol and the isomeric 2-hexene and have been obtained by total synthesis. They are not bound to the cytoplasmic estrogen receptor in vitro. Compound *2* was inactive in dimethylbenzanthracene-induced hormone-dependent mammary carcinomas of the rat (1 mg/rat sc, 6 days/week daily × 6 weeks).



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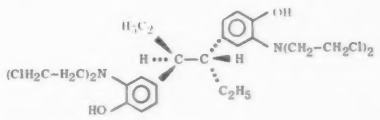

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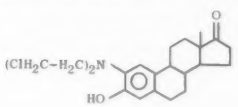
The nitrogen mustard derivatives *3* and *4* with two free hydroxyl groups have been synthesized from diethylstilbestrol and hexestrol, respectively. The binding affinity to the cytoplasmic estrogen receptor of *3* and *4* is 10^4 times lower than that of estradiol-17β. In contrast to nitrogen mustard derivatives, in which the alkylating function is linked to an estrophilic carrier by an ester group, *3* and *4* are irreversibly bound to the estrogen receptor. Both compounds, as well as *1* and *2*, do not exhibit any estrogenic activity in the Allen-Doisy test. When administered ip to mice, *3* and *4* exhibit an LD50 of 20 mg/kg and 11 mg/kg, respectively.



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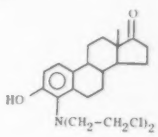

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As expected, the introduction of only one nitrogen mustard function close to the hydroxyl group of an estrophilic carrier results in a smaller decrease in receptor affinity related to the corresponding carrier molecule itself. Compounds *5* and *6*, synthesized by nitration of estrone and subsequent transformation of the resulting 2- and 4-nitroestrone into the corresponding nitrogen mustards, are irreversibly bound to the cytoplasmic estrogen receptor. Both compounds exhibit a binding affinity of 1%–10% relative to that of the carrier estrone itself. The preliminary results of the described estrogens

[1]Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

[2]This reported investigation was done with the cooperation of the groups of Drs. J.-C. Heuson and G. Leclercq of the Institut Jules Bordet, Brussels, Belgium. All biologic tests were conducted in the laboratory in Brussels.

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linked with alkylating groups and containing free hydroxyl functions are encouraging with respect to their potential use in the treatment of estrogen receptor-positive tumors.

Steroid Hormones Linked With Cyclophosphamide

Robert T. Blickenstaff, Veterans' Administration Hospital, Indianapolis, Indiana

Derivatives of estrone and dehydroepiandrosterone were synthesized as model compounds representing the covalent attachment of cyclophosphamide to steroids (1). As illustrated in figure 1, the cyclophosphamide ring was built onto carbon-17 of the D-ring of estrone methyl ether. The synthesis was extended to dehydroepiandrosterone in which the 3-hydroxyl group was protected as the tetrahydropyranyl ether; the cyclophosphamide ring of compound *8* was stable to cleavage of the tetrahydropyranyl ether linkage. The three steroidal cyclophosphamides as well as eight of the intermediates were screened against P388 lymphocytic leukemia and L1210 lymphoid leukemia in mice by the National Cancer Institute, and all were found to be inactive; these systems were chosen for the initial screening because cyclophosphamide is active in them. Subsequently, compound *7* was found to be inactive against Adenocarcinoma 755 and Gardner lymphosarcoma by Eli Lilly and Co.

Cyclophosphamide is known to undergo activation by enzymic hydroxylation at the 4 position. It was reasoned that an explanation of the inactivity of these steroidal cyclophosphamides may be that the hydroxylating enzyme may have sufficient substrate specificity as to be incapable of accommodating the bulky steroid moiety attached to the cyclophosphamide ring. Consequently, we proposed a chemical synthesis of the steroidal 4-hydroxycyclophosphamide 14 (fig 2). In practice, when intermediate *10* was treated with *N,N*-bis-(2-chloroethyl)phosphamide dichloride in refluxing benzene, no evidence for the production of intermediate *11* was obtained, and compound *10* was recovered unchanged. The question arises as to why the tert.-hydroxyl in *10* is unreactive while that in compound *6* is phosphorylated. It may be that with *6*, reaction occurs first at the amino group, after which ring closure is made easy by the proximity of the $-CH_2NHP(Cl)(O)N(C_2H_4Cl)_2$ group to the hydroxyl. The reader will recognize that the remainder of the proposed synthesis (11 → 14) follows the published synthesis of 4-hydroxycyclophosphamide (Takamizawa et al [2]). The work was curtailed at this point, but interest has been maintained in cyclizing intermediates like *15* and *17*, compounds which have been synthesized in our laboratory.

Affinity-Labeling to Evaluate Structure of Steroid Binding Sites on Enzymes and Receptors

J. C. Warren, Washington University Medical School, St. Louis

We have followed a strategy of developing steroid analogs with affinity-labeling reagent groups attached at various positions on the steroid nucleus. By carefully using several such compounds, one may hope to affinity-label sufficient numbers of amino acid residues in a given steroid binding site to elucidate its topography. The synthetic procedures reported are clean, straightforward, and reproducible. Since many of these syntheses are carried out on a semimicro scale, care must be taken if satisfactory yields of product are to be obtained. This is particularly true in the synthesis of tritiated derivatives, since products with a high specific activity are desired.

The importance of proof of absolute structure and purity of the final product cannot be overemphasized. If one were to use a derivative to which the wrong structural assignment had been made or one that had been contaminated with a second affinity-labeling derivative, confusing errors would be generated.

As is obvious, we have primarily relied on bromoacetoxy and bromoacetamido derivatives in our affinity-labeling studies. These compounds are reasonably stable, yet possess considerable reactivity with nucleophilic groups commonly found in amino acid side chains. The chloroacetoxy derivatives, on the other hand, are reasonably unreactive, and iodoacetoxy derivatives lack the stability of the bromoacetoxy compounds.

Influence of Steroidal Alkylating Agents on the Growth and Estrogen Binding Capacity of the R3230AC Mammary Carcinoma of the Rat

James L. Wittliff, Noel Weidner, Daniel C. Park, Richard B. Everson, and Thomas C. Hall[3]

Estradiol mustard and estramustine phosphate (Estracyt) are steroidal alkylating agents comprised of an estradiol-17β nucleus with cytotoxic side

[3]The University of Louisville School of Medicine, and the Cancer Center, Louisville, Ky (*J. L. Wittliff and D. C. Park*); Barnes Hospital, St. Louis, Mo (*N. Weidner*); National Cancer Institute, Bethesda, Md (*R. B. Everson*); and Huntington Memorial Hospital, Pasadena, Calif (*T. C. Hall*).

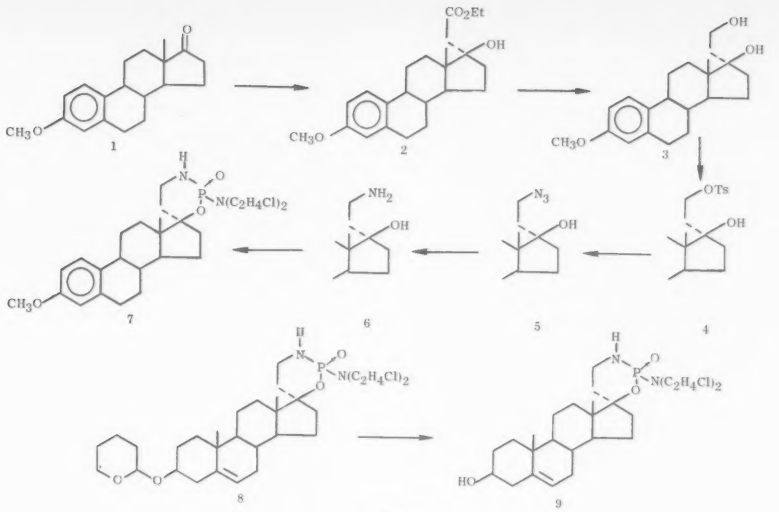

FIGURE 1

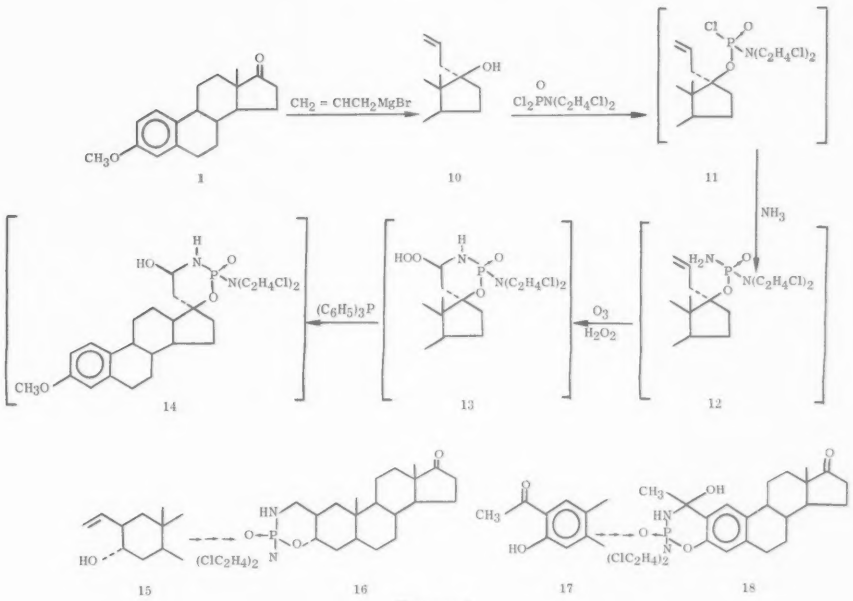

FIGURE 2

groups at carbon 3 or 17 or both. The former was synthesized and described by Wall et al (3) and the latter, estramustine phosphate, was produced by A. B. Leo of Helsingborg, Sweden.

Both agents were designed to combine the advantages of estrogens and cytostatics, although preliminary clinical trials have been conducted with variable therapeutic success in human breast and prostate carcinomas. In these studies, we investigated the influence of these steroidal alkylating agents on the growth of the transplantable R3230AC mammary adenocarcinoma of the rat. Previously, we found that this tumor contains specific estrogen receptors from studies in vitro (4) and in vivo (5).

Tumor implants of 10–20 mg were placed along the axillary-inguinal line of female Fischer 344 rats using a sterile trocar technique. Seven to ten rats each were placed in the treatment groups shown in table 1. On Day 21 after tumor implantation, ani-

TABLE 1.—Effect of steroidal alkylating agents on R3230AC tumor growth*

Treatment	Dose (mg/kg/day)	Tumor weight (g)	Treated/control
	Experiment 1		
Vehicle	—	1.25 ± 0.22	—
Phenester	2	0.79 ± 0.27	0.63
Estradiol valerate	1	1.03 ± 0.19	0.82
Estradiol mustard	5	0.71 ± 0.22	0.57
	10	0.58 ± 0.10	0.46
Estradiol valerate + phenester	1 2	0.16 ± 0.05	0.13
	Experiment 2		
Vehicle	—	4.79 ± 0.59	—
Estradiol valerate	1	4.23 ± 0.27	0.88
Estramustine phosphate	10	3.05 ± 0.51	0.64
Estradiol valerate + estradiol phosphate	1 10	3.46 ± 0.70	0.72

*A single implant was used in experiment 1 and multiple implants were used in experiment 2.

mals were killed and the tumor was measured. The estrogen binding capacity was measured using a dextran-coated charcoal procedure (6).

As seen in experiment 1 (table 1), administration of the drug combination, estradiol valerate and phenester (components of estradiol mustard), produced the greatest inhibition of tumor growth. However, there was a significant decrease in tumor weights in animals treated with estradiol mustard itself. Estramustine phosphate alone (experiment 2) inhibited growth of the R3230AC tumor but not to the extent shown by an equivalent dose of estradiol mustard. Uterotropic effects were seen in all groups given estradiol valerate as well as the steroidal alkylating agents (7).

Earlier, we demonstrated (8) that either estradiol mustard or a cleavage product inhibited the binding of [^{3}H]estradiol to receptors in uterine cytosol. Therefore, we investigated the influence of one of these agents, estramustine phosphate, on the estrogen binding capacity of the R3230AC tumor; the results are shown in table 2. These preliminary results suggest that the steroidal alkylating agent increased the level of estrogen receptors in this tumor. This induction apparently did not result from hydrolysis of the agent with production of estradiol since the estradiol valerate-treated animals exhibited no increase in estrogen binding capacity.

We conclude that steroidal alkylating agents such as estradiol mustard or estramustine phosphate exhibit antitumor effects on the growth of the R3230AC adenocarcinoma of the rat. We were unable to determine if this action was mediated by combining with intracellular binding sites which transported them into nuclei. Our results suggested further that estrogen and alkylating agent combinations may have positive synergism in the treatment of mammary cancer.

TABLE 2.—Estrogen binding capacity of R3230AC tumors

Treatment	[^{3}H]estradiol binding capacity (fmol/mg of cytosol protein)
Vehicle	12.1 ± 1.8
Estradiol valerate	12.0 ± 1.7
Estramustine phosphate	23.8 ± 3.5
Estramustine phosphate + estradiol valerate	28.3 ± 4.6

Interactions Between Estrogen Receptors and Certain Steroidal Alkylating Agents

James L. Wittliff, Noel Weidner, Richard B. Everson, and Thomas C. Hall[4]

Steroidal alkylating agents are known to influence the growth of a number of animal tumors (3,7,9) and human prostatic carcinomas (10). The potential of estrogen binding proteins in the cyto-

[4]*See* footnote 3.

plasm to mediate the action of the steroidal alkylating agents, estradiol mustard and estramustine phosphate, was investigated using competition studies of these drugs for specific binding sites on the receptor protein.

Cell-free preparations of receptors from either virgin rat uteri or from lactating mammary glands of females 14–21 days postpartum were made using 10 mM tris-HCl buffer, pH 7.4 or 8.0, containing 1.0 mM EDTA, 250 mM sucrose, and 10 mM monothioglycerol. Inhibition of ^{3}H-estradiol-17β binding by the steroidal alkylating agents was investigated by simultaneous incubation of various concentrations of the competitors with a constant concentration (usually 5 nM) of labeled steroid at 0°C at 14–16 hours. Residual binding was estimated by the dextran-coated charcoal procedure (6). Using the relationship suggested by Rodbard (11):

$$K_d\,(\text{competitor}) = K_d(^3\text{H-ligand}) \times \frac{I_{50}(\text{competitor})}{I_{50}(\text{unlabeled ligand})}$$

it was possible to estimate the dissociation constant (K_d) of the unlabeled ligand-receptor complex. The K_d of ^{3}H-estradiol-17β binding was determined separately by titration analyses.

Estradiol mustard inhibited the specific binding of ^{3}H-estradiol-17β to uterine cytosol receptors while the alkylating moiety, phenester, was without effect (8). Inhibition of binding by estradiol mustard was observed at $> 10^{-6}$ M and was complete at 10^{-3} M. From these data, an I_{50} of 3×10^{-5} M was determined giving a K_d of 3×10^{-6} M for the estradiol mustard-receptor complex assuming no degradation. Sucrose gradient analyses indicated that estradiol mustard blocked the formation of the 8S form of estrogen receptors in cytosol of uteri. Displacement of ^{3}H-estradiol-17β by estradiol mustard using cell-free preparations of receptors did not appear to be due to hydrolysis of estradiol mustard under the conditions utilized.

Using receptor preparations from lactating mammary glands, the K_d of the estramustine phosphate-receptor complex was 2.6×10^{-6} M compared to a K_d of 2.5×10^{-10} M for diethylstilbestrol. The K_d of ^{3}H-estradiol-17β determined separately by titration was 4×10^{-10} M. Inhibition studies using limiting concentrations of either estramustine phosphate or diethylstilbestrol and double reciprocal plots of the data indicated that each was a competitive inhibitor of ^{3}H-estradiol-17β binding. The K_d of estramustine phosphate estimated in this fashion was $1.7–5.5 \times 10^{-6}$ M. Sucrose gradient centrifugation indicated that the steroidal alkylating agent inhibited ^{3}H-estradiol-17β binding to the 8S form of the estrogen receptor in lactating mammary glands in a concentration-dependent fashion. The time course of inhibition studies suggested that at 0°C, some breakdown of estramustine phosphate occurred possibly to the dephosphorylated form. Earlier, Fredholm (9) found that estramustine phosphate was concentrated in vivo in dimethylbenzanthracene-induced mammary tumors as well as in the prostate and ovaries of rats. The majority of the agent was determined to be in dephosphorylated but otherwise intact forms.

The data presented here and in our other study (*see* previous section), do not resolve the fate of estradiol mustard and estramustine phosphate either in vivo or in vitro. Although apparent affinities for the estrogen receptor are low and some hydrolysis may occur in vivo, the antitumor effects of these steroidal alkylating agents suggest that a second generation combination of estrogens and cytostatics is in order.

Steroid Carriers of Cytotoxic Agents: Evaluation of Antineoplastic Effects of Drugs and Animal Models

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It is important to match the animal model to the drug being studied. Dr. Blickenstaff used P388/L1210 leukemias, and Dr. Gardner used cell cultures of human epidermoid cancers. These might have been suitable for the study of simple alkylating agents, but the present knowledge of tumors which are more apt to contain steroid receptors and which have responsiveness to hormones might now permit a better choice of animal models. It might now be possible to include the adenocarcinomas of breast and prostate in rodents.

Similarly, Dr. Leclercq suggested that his agents might work by receptor destruction by alkylation. The possibility of alkylating actors might be studied specifically by using these agents against animal tumors such as human epidermoid cancer, lymphosarcoma, or L1210 leukemia which are alkylating-sensitive.

Probably in future in vivo tests on animal models, any agent thought to have steroid and alkylating agent actions should be tried against animal models known to show sensitivity to both these classes of agents when used singly.

Models Which Test Hypothetic Concepts

The above comments underlie our use of the individual components of estradiol mustard against the hormone-sensitive, steroid receptor-positive rat mammary cancer R3230AC (table 3). This study showed that it was possible to achieve a superior anticancer effect by adjusting the proportions of

TABLE 3.—Effects of treatment in vivo of R3230AC mammary carcinomas of the rat with estradiol mustard or its molecular components

Group	Drug	Dose		No. of survivors/ total No. of rats treated	Body weight difference (g)	Uterine weight (mg)	Tumor weight (g)	Treated/ control*
		mg/kg/day†	μmol/kg/day					
A	None	—	—	7/7	+18	274±22‡	1.25±0.22‡	—
B	Phenester	2.0	7.27	7/7	+19	253±20	0.79±0.27	0.63
C	Estradiol valerate	1.0	2.78	7/7	+7	807±33	1.03±0.19	0.82
D	Estradiol mustard	5.0	6.35	7/7	+22	451±32	0.71±0.22	0.57
E	Estradiol mustard	10.0	12.69	7/7	+16	384±10	0.58±0.10	0.46
F	Estradiol valerate + phenester	1.0 2.0	2.78 7.27	7/7	+3	707±42	0.16±0.05	0.13
G	Estradiol valerate + phenester	2.3 3.5	6.35 12.69	3/7	–7	750±41	0.07±0.02	0.06

*Data represent mean ± SEM for all surviving animals in that group as recorded on Day 21.
†Total dose calculated on a per day basis was then given twice weekly.
‡Tumor weight in treated group/tumor weight in control group.

estradiol valerate and phenester than was possible in the case of the fixed 2:1 proportion, found in the intact molecule (7).

We also attempted to use this model of steroid receptor-positive breast cancer to determine whether the compound reacted directly with the 8S receptor or was first hydrolyzed into a free estrogen. This was difficult because of the lack of labeled compound, and the experiments should be done over, now that labeled compounds are available (8).

Models of the Clinical Situation

The clinical use of anticancer agents is often first required when a deep-seated metastasis appears years after the first resection of the primary. Thus, there is a need for a method for determining whether a new agent might be taken up by the deep metastasis, but also a method which avoids the need for major surgery to obtain tissue for studies of steroid binding.

Also, there is now increased realization that extensive surgery for breast cancer is useful primarily for diagnosis and staging rather than for treatment. Hence, smaller and smaller biopsies are being taken of large lesions, and complete mastectomies are now thought only justified in the cases with very small primaries and negative nodes. In neither instance is there much tissue left over for pharmacologic analysis after the pathologist is through. This increases the need for a noninvasive test for the presence or absence of steroid receptors.

At the University of Southern California we used the estrogen receptor-positive, R3230AC, and estrogen receptor-negative, NF13762, rat mammary cancers to estimate the relative uptake in vivo by tumor of ^{131}I radiolabeled estradiol. Thus, the tumor/blood and tumor/muscle ratios at 24 hours for the R3230AC tumor were 5.12 and 22.40, respectively, as compared with 0.16 and 1.85 for the estrogen receptor-negative NF13762. In addition, we have used the whole rat model to see whether uptake was sufficient in a limb-implanted lesion to permit external monitoring by scintillation scanning of ^{131}I. Substantial differential uptake was present between tumor and normal tissue; this was true in the estrogen receptor-positive tumor primarily, and the rat model identified the site of leg implantation of the positive tumor.

Clinical Experience With Estramustine Phosphate and Prednimustine, Two Steroidal Alkylating Agent Compounds

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The clinical experience with steroid-alkylating agent compounds in breast cancer is limited (12,13). However, their use in prostatic cancer was more extensively studied, and a review of the results obtained at Roswell Park Memorial Institute (Buffalo, NY) is presented.

Estramustine phosphate (fig 3) was given orally in a dose of 15 mg/kg/day continuously without serious side effects. No hematologic toxic effects were encountered and the dose-limiting factor was gastrointestinal intolerance and occasional fluid accumulation. Fifty patients with advanced, hormone-resistant prostatic cancer were treated and 36% of them improved while receiving therapy. In half of

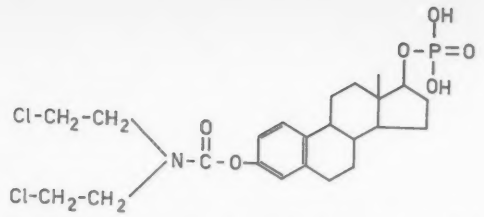

FIGURE 3.—Estramustine phosphate (Estracyt): estradiol phosphate carrying a nitrogen mustard derivative, linked to C3.

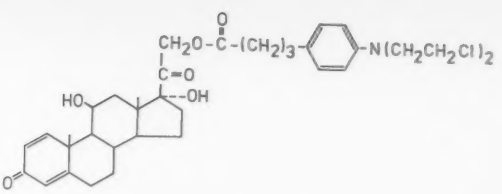

FIGURE 4.—Prednimustine (Leo 1031): prednisolone carrying chlorambucil, esterified at C21.

these patients (19% of the total No. of patients) signs of objective tumor regression could be documented (14). Prednimustine (fig 4) given orally at a dose of 25 mg/m²/day continuously in 25 patients (also with hormone-resistant advanced prostatic cancer) gave a 35% response rate with 13% objective regression. The dose-limiting toxic effect of this compound was myelosuppression.

A combination of both agents gave a slightly higher response rate: 44% improved and about half of them (24% of all patients) had objective tumor regression (15).

The mechanism of action of these drugs in prostatic cancer is now being intensively studied (16–18).[5] The future usefulness of these and similar agents in the treatment of hormone-dependent cancers will have to be established by comparative studies with cytotoxic agents without steroid carriers (19), and will depend in large part on understanding the factors responsible for therapeutic effects.

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Problems in the Development of Cytotoxic Moieties Linked to Hormones

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The proper evaluation of currently available steroid drugs as carriers of cytotoxic moieties and the development of new agents will require considerable dialogue between the synthetic chemist, the tumor biologist, the molecular endocrinologist, and the clinician. This discussion may be regarded as an initial step in this dialogue.

The following aspects need to be considered for both currently available and new drugs that are being synthesized or designed:

1. Mechanism of Action for Cytotoxicity and Specificity

Two possible mechanisms of action are conceivable. First, the antitumor effect could be exclusively dependent upon binding of the drug to the hormone receptor. Inactivation of the receptors following binding could lead to cell dysfunction; in addition, binding to the receptor could be the basis for tissue or tumor specificity of the drug. In this sense, the drug would be acting as a potent "antihormone." However, it is realized that this concept is currently totally speculative and likely to be oversimplified. Another possibility, perhaps more attractive, is that the alkylating moiety is still available for cytotoxic actions as the drug is transported while bound to the receptor towards the cell nucleus and other vulnerable sites, thereby causing destruction of the cell. Both mechanisms require a priori that the receptor specificity of the new compound be retained, and that the cytotoxic moiety not be split off or inactivated before it reaches its target. Radioactive labeling of the compound may be helpful in gaining more information along these lines.

Experiments probing the validity of these theoretic concepts are needed. Even more urgent, however, is the development of systems which select and identify optimal compounds with the desired properties (ie, receptor binding) and which indicate that these might be biologically active against hormone-dependent tumors.

2. Assays for Receptor Binding Affinity

In the synthesis of these drugs, it is apparent that their receptor binding affinity and metabolism are of central importance. Several important "nuances" in these studies require attention: (*a*) "irreversible binding" of the drug to receptor by competitive binding cannot be distinguished from actual destruction of the receptor; (*b*) estrogen receptors from various tissues may have different specificities or subcellular localization; (*c*) the methods of defining receptor affinity always need to be stated, whether deduced from direct binding or competition experiments (the latter type of experiments are subject to many potential errors); and (*d*) at high drug concentrations, nonspecific effects may interfere with assays (this is particularly true if the addition of a cytotoxic moiety to the hormone leads to reduced binding affinity). Also, notwithstanding receptor specificity, it must be recognized that mechanism of action may be totally unrelated to this property. Thus, testing in appropriate biologic systems is always essential.

3. Assessment of Antitumor Activity

Human cell lines which have been well-characterized in their receptor content and hormonal dependence should be the first biologic systems in which to test new drugs of this type (*see* below). Although there may be important differences between cell lines and the in vivo situation, they should represent a useful initial screen. If these cell lines can grow as tumors in nude mice, such in vivo studies would represent a logical and attractive subsequent step in the evaluation. This system would provide a measure of antitumor activity, allow comparison with hormonal and nonhormonal cytotoxic agents, and indicate other pharmacologic and toxicologic properties of the agents.

Other "conventional" antitumor screening models may be mentioned. In many drugs evaluated in the past, the appropriate controls were not done. For example, activity of a drug should always be com-

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pared with the action of the steroid carrier alone and the cytotoxic agent alone. However, unless a system such as the one indicated above is used, the antitumor effect in vivo may bear no relationship to the affinity of the drug for the receptor.

4. Use of Human Breast Cancer Cell Lines in Continuous Tissue Culture for the Evaluation of Cytotoxic Agents Linked to Hormone Carriers

There are many advantages to the use of human breast cancer cell lines to screen a number of hormone-cytotoxic agent conjugates. First, a great deal of unequivocal data have established the human and mammary nature of these cell lines. While considerable cell selection may accompany the adaption of cells to continuous culture conditions, responsiveness to various hormonal factors has been retained. Second, these cell lines have been shown to have receptors for estradiol, progesterone, glucocorticoid, androgen, insulin, and triiodothyronine. Extensive characterization of the properties of the estrogen receptor from these cells has shown it to be indistinguishable from that detected in fresh malignant material. Thus, these cells provide a highly suitable vehicle for the study of the interaction of a variety of liquids with receptors. Third, tissue culture systems are well suited for the study of drug metabolism. Following suitable incubation periods, cells and medium are readily analyzed for metabolites of the compound of interest. Such studies can aid greatly in attributing observed activity to the appropriate source. Fourth, by growing cells in the presence of the compounds of interest one may be able to select drug-resistant variants. Biochemical and genetic analysis of these resistant cells may provide significant insight into the mechanisms of drug action. Fifth, variant cell lines (derived from parental clones which are receptor-containing and estrogen-responsive) which have been selected in the presence of antiestrogens can be obtained which have lost estrogen receptor activity. Such cells provide a uniquely advantageous control for separating out those effects of hormone-cytotoxic agent combinations which are receptor-mediated (and therefore of particular interest) from those which are less specific.

CONCLUSIONS

New compounds, and also old compounds which have been synthesized or have even received clinical trial, should be evaluated in previously described test systems which may indicate their receptor affinity and biologic activity. Although these systems may provide relatively simple ways of screening for new agents, the mechanism of action of compounds already available should also be more closely defined. Breast cancer cell lines and estrogen receptor assays have been the subject of intensive studies; these represent excellent tools with which to explore these concepts. Such lines of investigation will perhaps ultimately yield cytotoxic drugs with greater potency and more specificity for human breast cancer than have been available until now.

Finally, these concepts may be extended to the treatment of other tumors which also possess estrogen receptors. Moreover, work on other carrier molecules may be similarly approached in the future. It is hoped that this workshop has provided some basic guidelines for such subsequent investigations.

LETTERS

Hypertensive Reaction to Adriamycin

The clinical toxicity of adriamycin is by now fairly well known. Toxic effects include myelosuppression, mucositis, alopecia, phlebitis, hyperpigmentation of nail beds and dermal creases (1), recall skin reactions in previously irradiated areas (2), and facial flushing occurring on rapid drug administration (3). Gastrointestinal toxic effects such as nausea and vomiting are particularly frequent, and may be associated with anorexia and diarrhea. A hypersensitivity reaction with fever, chills, and urticaria has been reported, and conjunctivitis and lacrimation can occasionally occur (4). Cardiotoxicity is a major problem and the cardiomyopathy is related to the total dose (5).

A most unusual reaction to adriamycin which is reported here consisted of lacrimation, headache, drowsiness, and on occasion, loss of consciousness associated with a marked rapid rise in blood pressure shortly after an iv bolus of adriamycin.

CASE REPORT

The patient is a 69-year-old woman with metastatic carcinoma of the breast with the dominant site of disease in the bone. She was treated by simple mastectomy followed by postoperative radiotherapy in 1973 for an infiltrating ductal carcinoma of the right breast. She was well until 1975 when she developed a local recurrence which was surgically excised. By April of the following year (1976) she had developed metastases in the bone and lungs. She received ethinyl estradiol (0.5 mg twice daily) but was unable to tolerate estrogen therapy. In June of 1976 the estrogens were stopped and she then received cyclophosphamide, methotrexate, and 5-fluorouracil due to increasing bone pain. Following two full-dose courses of this chemotherapy, it was apparent that she was not improving, and at the end of August 1976 she was given adriamycin. The patient lived in a remote part of the province of Alberta, Canada, and her family practitioner administered the adriamycin chemotherapy (80 mg iv every 3–4 weeks). He reported that the patient appeared to be doing well with a great reduction in her pain but that a problem had developed in that when she was given iv chemotherapy she became unconscious, and upon waking, complained of severe chest pains, some breathlessness, and headache. This passed very quickly and in fact, chest roentgenogram, cardiogram, and cardiac enzymes were normal and she was discharged. The same phenomenon occurred following the second injection of adriamycin and the practitioner described the event as similar to "giving an iv general anesthetic." Subsequent courses with a reduced dose of the drug led to drowsiness but not complete loss of consciousness.

She was admitted to the Cross Cancer Institute in Edmonton (Alberta, Canada) for investigation of the attacks and administration of adriamycin. She denied any previous history of epilepsy or similar attacks, and a physical examination was unremarkable. The patient was scheduled for an iv injection of 60 mg of adriamycin. She was attached to a cardiac monitor with the emergency trolley standing by. A resident measured her blood pressure and pulse rate. Another resident took blood samples for calcium, glucose, and electrolytes and two other physicians were observers. Her blood pressure prior to the injection was 150/90. Five minutes following the injection, the patient said "an attack is coming on." She was observed to tense in all muscle areas and there was a tremor on the right side of the face. She was lacrimating and she complained of headache. An ophthalmoscopy at this time was normal. Her pulse rate slowed, however, and the blood pressure rose rapidly to a maximum of 220/160 8 minutes following the adriamycin injection and gradually returned to normal over the next 15 minutes. Her clinical condition improved with the fall in blood pressure. Calcium, glucose, and electrolyte levels were normal before and during the attack. The following day, in view of the remote possibility that she had had an hysterical reaction, a placebo injection of "adriamycin" was given, using vitamin B12 which was suitably colored to resemble adriamycin. The patient was again attached to the cardiac monitor and all persons who had been present the previous day were present for the second injection. Following this injection, no response was noted and the patient remained well.

The patient therefore appeared to have hypertensive encephalopathy following the injection of adriamycin. This has not previously been reported to occur with this drug although vascular side effects such as flushing are known to occur. It was thought possible that the patient had an excessive secretion of catecholamines following injection of adriamycin,

possibly due to pheochromocytoma, but a 24-hour urine collection for vanillylmandelic acid was normal. Shortly after this admission, adriamycin was discontinued not only because of this dangerous side effect, but also because her metastatic disease was progressing radiologically. This elderly woman did not appreciate the clinical interest we had in her reaction and she resented being subject to so much investigation. She was subsequently treated with radiotherapy as and when pain developed with no systemic therapy and has been well over the last 6 months. In the last month we have started giving her tamoxifen with no ill effects so far.

In conclusion, this patient had a good response to adriamycin with healing of bone lesions but appeared to have a hypertensive crisis associated with transient encephalopathy each time an injection was given. We would be very interested in hearing whether this uncommon side effect has been seen elsewhere in patients receiving this widely used cytotoxic drug.

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Metronidazole in Cancer Therapy

An Alternate View

I should like to make the following comments on the paper by Frytak et al (1) concerning the use of metronidazole in cancer therapy. It has never been suggested that metronidazole has any antineoplastic potential as a single agent. The work of Sutherland et al and Mohindra and Rauth quoted by Frytak et al clearly demonstrates that metronidazole is active against only the hypoxic cell component of tumors. Thus, if any benefit is to be derived from the use of metronidazole in this way, it must be used in conjunction with another agent with activity against the oxic cell component of the tumor which in terms of volume may be expected to account for the greater part of the whole tumor. It is, therefore, hardly surprising that no objective tumor response to metronidazole therapy was observed, because even if every hypoxic tumor cell had been killed and removed from the tumor, the oxic tumor cells would continue to proliferate and add to the volume of the tumor (2,3).

Since the minimum effective concentration of metronidazole against hypoxic tumor cells and the optimum treatment time have not been determined under in vivo conditions, the clinical application of metronidazole in this role must be regarded as premature.

The use of high doses of metronidazole over a prolonged period of time in patients in a lowered nutritional state or in patients who have been treated recently with cytotoxic drugs is associated with an increased incidence of side effects (4). The side effects in these patients with advanced colorectal carcinoma were, therefore, predictable and in contrast to the trouble-free use of much higher doses of metronidazole reported by Urtasun et al (5).

Thus, the role of metronidazole in cancer therapy as suggested by Sutherland et al remains to be evaluated in patients.

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May 18, 1978

Author's Response

Dr. J. L. Foster is, of course, entirely correct in stating that our investigation of metronidazole had the long-term objective of using this agent in combination regimens. We would hope, however, that he would not be critical of evaluating this agent alone before approaching the intricacies of such combinations. The overall treatment benefit that could be derived from an agent effective in killing hypoxic tumor cells would be significant. One is seldom so fortunate as to encounter a single drug that will destroy the entire tumor cell population and, in the majority of solid tumors, chemotherapy cure is usually unlikely.

With regard to the question of toxicity, it might also be argued that the evaluation of a drug administered alone holds some advantage over trying to assess the contribution of a single agent to the overall toxicity spectrum of a multi-drug or -modality combination. One could conceive of some difficulty in assessing central nervous system toxicity in a patient with a brain tumor who has recently had brain surgery and is now receiving cerebral radiation as well as corticosteroids and possibly diphenylhydantoin (see, for example, the study of Urtasun [1]). The central nervous system toxicity encountered in our studies of metronidazole was certainly not appreciated previously. If there is a possible connection between nutritional status and central nervous system toxicity associated with metronidazole therapy as implied by Dr. Foster, the therapeutic application would be greatly curtailed since many tumor patients unfortunately have an altered nutritional status. Our patients had to sustain a minimum caloric intake of 1200 calories before entering the study.

We must hold that evaluation of single drugs or modalities is an appropriate, if not mandatory, first step in laying the foundations for their use in combination therapy.

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June 19, 1978

Pulmonary Reaction Associated With Neocarzinostatin Therapy

Neocarzinostatin (NCS) is an antineoplastic antibiotic currently in experimental use. It is a single-chain polypeptide with a molecular weight of 10,700 and a known primary structure (1).

Experimentally, Schaeppi et al (2) noted toxic reactions to NCS in dogs in two broad categories: radiomimetic and immunologic. Side effects of NCS used in man have been minimal (3). No pulmonary toxicity from the drug has been described. We report here a case of an acute pulmonary reaction associated with NCS therapy.

The patient was a 19-year-old male who in March 1973 was found to have a multilobulated hepatocellular carcinoma of the right lobe of the liver after an extensive workup for fever of an unknown origin. Following a right hepatic lobectomy, 5000 rads of radiation were given to the right upper quadrant over a 35-day period. The patient did well until October 1976 when relapse was discovered in the scrotum, rectum, and peritoneum. NCS therapy was begun in October 1976 at a dose of 2250 units/m^2/day iv $\times$ 5 days. He received five courses of this therapy until February 15, 1977, with no ill effects except for transient thrombocytopenia. The rectal mass decreased in size. No other chemotherapeutic agents were given.

Approximately 1 month after the last dose of drug the patient developed a dry cough. He was afebrile but arterial blood gases were abnormal with a PO_2 of 55 mm Hg, and a chest roentgenogram at this time revealed a disseminated, coarse interstitial pattern not unlike interstitial edema. Noninvasive diagnostic evaluation including multiple sputum analyses and cultures, serum and urine electrophoresis, and complement assay did not yield a diagnosis. Binding of ^{125}I-labeled NCS by the patient's serum was assayed by a double-antibody technique. In contrast to normal human serum, a low level of binding activity was detected in a 10-μl aliquot. No binding activity was detected in 1 and 0.1 μl of the patient's serum. Similar binding activity was found in two other patients who had received multiple courses of NCS but had no evidence of lung disease. An open-lung biopsy showed an unusual pulmonary lesion characterized by endothelial edema and endothelial cell hypertrophy of pulmonary arterioles and venules with an associated small venous thrombus. There was interstitial edema and early fibrosis of interlobular septa. Bacteriologic and viral cultures and special stains for mycobacteria, fungus, and parasites failed to demonstrate a causative organism.

The patient was given 60 mg/day of prednisone with clinical and radiographic improvement. During convalescence, a spontaneous thrombosis of the left axillary vein occurred, and the patient was discharged and given Coumadin therapy. Two weeks later he was re-admitted because of hemoptysis and died from rapidly progressive pulmonary insufficiency.

At post-mortem examination, the pulmonary lesion had progressed to diffuse vascular wall thickening with prominent intimal thickening of the arterioles and mild interstitial fibrosis. Special stains and cultures again yielded no etiologic agent. Immunofluorescence of lung tissue was negative, and electron microscopy failed to show deposits in the alveolar basement membranes. These lesions and their progression, though not specific, were morphologically identical to the bleomycin-induced pulmonary fibrosis described by Adamson et al (4) in mice. A post-mortem study of the kidneys also showed endothelial cell injury manifested by cell loss in the glomerular capillaries and scattered fibrin thrombi.

The pulmonary toxicity of cytotoxic drugs has been recently reviewed (5). The pulmonary reaction associated with NCS therapy in this patient shared clinical, radiographic, and pathologic features with reactions to these agents, particularly bleomycin. The patient received an unusually large amount of NCS, the largest dose of any of the 100 patients receiving the drug at the Dana Cancer Center; perhaps the drug exerted a direct toxic effect on the vascular endothelium. The patient also may have developed precipitating antibodies to the drug (as did dogs given NCS experimentally; ref 2), perhaps indicating the presence of an immunologically mediated process. It is possible that additional instances of lung toxicity will be observed as larger dose levels of NCS are achieved.

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May 31, 1978

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ANNOUNCEMENTS

Seminars on Oncology at Roswell Park Memorial Institute

Buffalo, New York

There will be a seminar on "Current Trends in Analgesia for Cancer Patients" on November 18, 1978, organized by the Department of Anesthesia at Roswell Park Memorial Institute. The chairperson is Dr. Raymond Trudnowski.

There will be a $25.00 registration fee. *For further information contact:* Ms. Claudia Lee, Cancer Control, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

First International Congress on Cardiovascular Surgery and Diseases

Las Vegas, Nevada, January 14–19, 1979

Drs. Michael DeBakey and Eliot Corday will conduct the new world-wide congress on cardiovascular diseases and surgery, to be held jointly with the American Society of Contemporary Medicine and Surgery at Caesars Palace, Las Vegas, Nev, January 14–19, 1979. Participants may receive up to 40 hours of CME credit in Category 1.

For information, write: Rutledge Howard, MD, Director of CME, American Society of Contemporary Medicine and Surgery, 6 N Michigan Ave, Chicago, Ill 60626.

Chemotherapy Foundation Symposium III: "New Developments and Changing Concepts in Cancer Chemotherapy"

New York City, New York, October 27 and 28, 1978

A concentrated 2-day symposium on cancer chemotherapy to be held on October 27 and 28, 1978, at the Barbizon Plaza Hotel in New York City introduces many new developments and changing concepts in cancer chemotherapy.

Chemotherapy Foundation Symposium III participants will (*a*) survey the progress in breast cancer adjuvant chemotherapy and the improved control of ovarian cancer with polychemotherapy; (*b*) report on the long-term control of testicular cancer, the second generation of adriamycin derivatives under investigation, the current status of platinum compounds, and the newer botanicals used in chemotherapy, and (*c*) provide an analysis of the European and American experience with podophyllin derivatives.

Presented under the joint sponsorship of the Division of Medical Oncology, the Department of Neoplastic Diseases, and the Page and William Black Post-Graduate School of the Mount Sinai School of Medicine, the 32 speakers include leading clinicians and researchers from the US and abroad. The Page and William Black Post-Graduate School of Medicine certifies that this continuing medical education offering meets the criteria for 14 Category I credits towards the Physician's Recognition Award of the American Medical Association provided it is used and completed as designed.

Registration fees are $150 for physicians and $50 for housestaff, fellows, students, and other health-care professionals. *For complete programs and registration information contact:* Dr. Ezra M. Greenspan, Symposium Chairman, Division of Medical Oncology, Mount Sinai Hospital, 1 Gustave L. Levy Pl, New York, NY 10029 (telephone, 212–650-6772).

23rd Annual Clinical Conference on "Cancer of the Genitourinary Tract"

Houston, Texas, November 2–3, 1978

The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute in Houston, announces the 1978 Clinical Conference on "Cancer of the Genitourinary Tract" to be held at the Shamrock Hilton Hotel, Houston, Texas, November 2–3, 1978. This Conference will focus on issues of diagnosis, staging, and therapy as they relate

to the critical and sometimes controversial therapies of the multiple stages of urologic malignancies. The Conference will be jointly sponsored by The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, and the American Cancer Society, Texas Division, Inc. It will provide practicing primary care physicians, oncologists, and urologists with critical issues at the moving edge of therapeutic investigation on urologic malignancies. An opportunity will be provided for speakers and registrants to exchange information at the conclusion of each session. There will be a registration fee.

This medical-education offering meets the criteria for 14 hours in Category I of the Physician's Recognition Award of the American Medical Association. This program is also acceptable for 14 prescribed hours by the American Academy of Family Physicians.

For additional information write: George R. Blumenschein, MD, Office of Education, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Tex 77030.

Workshop on Cloning Human Tumor Stem Cells

Tucson, Arizona, January 3–5, 1979

A practical workshop on soft agar cloning methods for human tumor stem cells will be held at the University of Arizona College of Medicine in Tucson, January 3–5, 1979, under the sponsorship of the Cancer Center Division. The workshop will be presented by Drs. Sydney E. Salmon, Anne Hamburger, David S. Alberts, Ronald N. Buick, and colleagues.

The registration fee is $150.00. *Further information and registration forms may be obtained by writing to:* Cancer Center Division, University of Arizona, Health Sciences Center, Tucson, Ariz 85724, or by phoning Dr. Ronald Buick at (602) 626-6408.

14th Annual San Francisco Cancer Symposium on "Body Image, Self-Esteem, and Sexuality in Cancer Patients"

Hyatt on Union Square, San Francisco, California, March 23 and 24, 1979

The West Coast Cancer Foundation announces the 14th Annual San Francisco Cancer Symposium on "Body Image, Self-Esteem, and Sexuality in Cancer Patients" to be held in San Francisco, California, on March 23 and 24, 1979. Registration fees range from a maximum of $125 to a minimum of $10 (with the lower fees for registrants applying before December 1, 1978). *For further information contact:* Jerome M. Vaeth, MD, West Coast Cancer Foundation, 50 Francisco St, Suite 200, San Francisco, Calif 94133 (telephone, 415-981-4590).

Symposium on "Sarcomas of Soft Tissue and Bone in Childhood"

Orlando, Florida, January 25–27, 1979

This symposium, sponsored by the Cancer Clinical Investigation Review Committee of the National Cancer Institute, will be held at the Holiday Inn in Orlando, Florida.

For further information, please contact: Dr. Arvin S. Glicksman, Department of Radiation Oncology, Rhode Island Hospital, Providence, RI 02902.

Second International Conference on the Adjuvant Therapy of Cancer

Tucson, Arizona, March 28–31, 1979

The Second International Conference on the Adjuvant Therapy of Cancer will be held in Tucson, Arizona, March 28–31, 1979, under the sponsorship of the Cancer Center Division of the University of Arizona. Drs. Sydney E. Salmon and Stephen E. Jones will serve as Conference Co-Chairmen. Abstract forms are now available; the deadline for submission of abstracts (prepared in the format of the American Association for Cancer Research/ American Society of Clinical Oncology) is December 1, 1978.

For abstract forms or further information, contact: Dorothy Baker, Conference Coordinator, Cancer Center Division, University of Arizona, Tucson, Ariz 85724 (telephone, 602–626-6044).

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